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SIMON FLEXNER, M D

PEYTON ROUS, M D

HERBERT S GASSER, M D

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NUTRITIONAL DEFICIENCIES AS A CAUSE OF ELEVATED BLOOD PRESSURE IN RATS (WITH ESPECIAL REFERENCE TO THE VITAMIN B₂ COMPLEX)*

By ROYALL M. CALDER, M.D.

(From the Clayton Foundation for Research, Houston)

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Many recent experimental observations are compatible with the idea that certain types of hypertension may be of metabolic origin and that the effective etiologic mechanism is diminished oxidative activity of the kidney. The close connection between some of the vitamins and metabolic processes suggests that vitamin deficiencies might cause a rise in blood pressure by limiting the oxidative capacity of the kidney. Such a possibility has been adopted as a working hypothesis in the present study, attention being given especially to deficiencies of vitamin B since this complex is known to furnish important components of several respiratory enzyme systems. The results indicate that a reduced intake of the entire complex is followed by a slight fall in the blood pressure of rats, whereas a deficiency of only the heat-stable portions of this complex leads to a reversible rise in blood pressure.

The discovery by Goldblatt (1) that constriction of the renal arteries is followed by persistent hypertension has been interpreted by some authorities as indicating that "arterial and arteriolar sclerosis of the kidney probably precedes and determines the existence of hypertension" (2). However the occurrence of even a few cases of essential hypertension without arteriolar changes and the observation that hypertension itself may cause arteriosclerosis (3) have kept alive the question of "whether arteriolar sclerosis in the kidney is cause or effect, or both, of hypertension" (2). The search for etiological factors which might cause hypertension by duplicating the physiologic consequences of renal ischemia and which might even lead to sclerosis of the arterioles cannot, therefore, be abandoned.

Within recent years, much experimental work has served to revive the old idea that essential hypertension may be a metabolic disease. Suggestive evidence, reviewed by Blalock (4), is to be had from the fact that the mediator of experimental hypertension is a blood borne chemical though whether the effective part of the mechanism is an excess of pressor substances or an insufficiency of anti-pressor substances has yet to be determined definitely. More direct evidence in favor of the possibility was supplied by Dicker (5), who showed that the formation of pressor

* The studies on which this paper is based were conducted with the support and under the auspices of the Clayton Foundation for Research. Laboratory space and other physical facilities were provided by the Duke University School of Medicine, whose generosity is gratefully acknowledged.

substances by the kidney is in no way related to its excretory functions. The older objections to the theory of the metabolic origin of hypertension were thereby eliminated, for these objections had been based mainly on the fact that the majority of patients with hypertension do not show any impairment of renal excretory functions (6). Finally, the essential metabolic nature of experimental renal hypertension has been firmly established by the work of Rodbard (7) and of Rodbard and Katz (8). According to these authors, the chemical mediator of hypertension is a normal product of renal intermediate metabolism which is usually destroyed as rapidly as it is formed.

If the latter interpretation be correct, then the problem resolves itself into a search for the mechanism by which ischemia might cause metabolic abnormalities in the kidney. A clue of possible profit in this search is the finding that constriction of the renal arteries leads to diminished consumption of oxygen by the ischemic organ, this has been demonstrated both in the intact animal (Levy, Light, and Blalock (9)) and *in vitro* by the manometric studies of Gerbi, Rubenstein, and Goldblatt (10). The possibility therefore arises that the unknown metabolic defect may be dependent upon inadequate oxidation in the kidney. This suspicion is somewhat strengthened by the observation of Holtz and Heise (11) that in the absence of oxygen the incubation of *L*-dihydroxyphenylalanine (dopa) with renal cortex extract results in the formation of a pressor amine. Bing (12) and Bing and Zucker (13) have noted a similar reaction on perfusion of ischemic kidney, the amount of pressor substance being inversely proportional to the perfusion rate. These authors believe that this pressor amine results from the incomplete oxidation of dopa, and that the reaction is the prototype of perhaps many such reactions, all of which may contribute to the hypertensive mechanism. In the presence of oxygen, this pressor amine is oxidized further to a non-pressor substance, presumably by oxidative deamination, a process which Kempner (14) has shown is impeded by low oxygen tensions.

The foregoing experimental observations admittedly would provide a plausible explanation of the hypertensive mechanism. Their acceptance as the actual explanation, however, necessitates the assumption that arteriolar or arterial sclerosis precedes the development of hypertension in every case, and, as stated above, there are valid objections against this assumption universally. In postulating that hypertension precedes the sclerosis, on the other hand, one is automatically abandoning organic interference with the blood supply as the primary cause of diminished oxidation in the kidney and is forced to look elsewhere for the explanation. Theoretically, one possible explanation hinges on the fact that biological oxidations are dependent not only on an adequate supply of oxygen but also on a multiplicity of enzyme systems the integrity of which is essential if oxidation is to proceed in a normal fashion. It is conceivable that the various oxidative functions of the kidney might be seriously compromised if there were a deficiency in these enzyme systems. This possibility is strengthened by the investigations of Schroeder and Adams (15), who demonstrated that tyrosinase has the capacity of inactivating renin and other pressor substances, as well as of combating experimental arterial hypertension.

In this connection, it is important to recall that certain components of these enzyme systems, such, for example, as the majority of the specific dehydrogenases, are intracellular and presumably an intimate part of the cell structure itself, others, such as the prosthetic groups of the di- and triphosphopyridine nucleotides and the flavins, are derived from exogenous sources. It is obvious that if these exogenous sources are cut off, the effectiveness of those systems involving coenzymes and flavoproteins must be reduced. The consequence would be a diminution of oxygen utilization by the body, including the kidney, and thus a condition would be created in the kidney duplicating that produced mechanically by the Goldblatt clamp.

This concept has provided a working hypothesis in the present study. Since the vitamin B complex is the source of several important components of the respiratory enzyme systems, it was chosen for the initial studies reported below. Analogous studies on a human subject, with similar results, have been reported by Elsom (16). The results of the present experiments are compatible with but by no means definitive proof of the working hypothesis just outlined.

Materials and Methods

Experimental Animals—Most of the rats used in these experiments were obtained from a breeding colony of the Vanderbilt strain (17). Those used in Experiment 2 were from a piebald strain obtained from the School of Hygiene, Johns Hopkins University. At the beginning of each experiment they varied in age from 8 to 10 weeks.

Diets—The rats were raised on a stock diet consisting of commercial dog chow checkers,¹ lettuce or other green vegetables, and white bread, and in addition received fresh milk for the 4 weeks following weaning.

The basic vitamin B free diet was that described by György and Goldblatt (18) and consisted of 18 parts of vitamin-free casein,² 68 of commercial sucrose, 8 of melted butter fat, 4 of U. S. P. salt mixture No. 2³ and 2 of cod liver oil. As indicated in the protocols various supplements were administered as follows: thiamin chloride in aqueous solution by dropper, in the amount of 40 μ gm. per rat per day, dried brewers' yeast⁴ either autoclaved or unautoclaved and in amounts as noted, crude aqueous extract of liver,⁵ 1 ml. per rat per day, a concentrated extract of rice polishings,⁶ 0.5 ml. per rat per day. The latter three ingredients were mixed with the foodstuff.

The term *vitamin B₂ complex* is used throughout this paper to denote the heat stable fractions of the vitamin B complex.

¹ Purina brand.

² Labco brand, The Borden Company, New York City.

³ S. M. A. Corporation, Cleveland.

⁴ Mead's brand.

⁵ Valentine Meat Juice Company, Richmond.

⁶ "Vitab" furnished gratis by National Oil Products Company, Harrison, New Jersey.

Laboratory Conditions—The rats were kept in roomy cages equipped with wire mesh bottoms to prevent access to the feces. Scrupulous cleanliness was maintained in order to avoid epidemics. During the summer months the laboratory was air-conditioned, the temperature being kept constant at 70–75°F.

Description and Discussion of Methods of Estimating Blood Pressure—The method described by Williams, Harrison, and Grollman (19) was used in estimating blood pressure. The readings so obtained are measures of mean pressures and are thus lower than the actual systolic levels. The method presented certain difficulties, arising from the fact that preliminary heating of the animals is required in order to produce a vigorous circulation through the tail. In general, the deficient rats require more heating for this purpose than do normal animals, but deficient animals withstand heating very poorly. Hence it was necessary carefully to standardize the duration of heating. In all except Experiment 1, the animals were handled in groups of four and were kept in the warmer box for 5 minutes and then transferred to the cooler box, from the latter, they were taken at random, and the readings on all four were completed within the ensuing 5 minutes. Only by strict adherence to these conditions can comparable pressure readings be obtained.

When large numbers of pressures were being determined, the temperature of the boxes of course varied somewhat, and since the boxes were opened frequently, thermostatic control was impractical. The varying temperatures were therefore compensated by alternating control and experimental groups of four animals with strict regularity.

It was found also that repeated heating of deficient rats has a rapidly cumulative effect. If pressure readings are attempted at too frequent intervals, many of the animals go into partial or complete collapse with resultant fall in arterial tension. Intervals of at least 3 days and preferably a week appear essential.

The accuracy of the above method of estimating blood pressures was checked by insertion of a cannula directly into the abdominal aorta, as described in Experiment 5. The findings indicate the reliability of the indirect method, provided the various precautions mentioned are observed meticulously.

EXPERIMENTAL

Influence of a Complete Deficiency of the Vitamin B Complex and of the Vitamin B₂ Complex on Blood Pressure

Experiment 1—One group of 50 rats was placed on a diet completely devoid of all components of the vitamin B complex, after 2 weeks on this regime, thiamin chloride was administered. A second group of 50 animals was placed on an identical diet, and autoclaved yeast was mixed with the foodstuff in the proportion of 5 per cent, after 2 weeks of this regime, the amount of yeast was increased to 10 per cent, and after 1 week of this treatment, the animals were subdivided into two groups of 25 each, one group receiving a supplement of liver extract and the other group receiving a supplement of extract of rice polishings.

The results of this experiment are shown in Figs 1 and 2. During the 1st week, the animals which received no vitamin B whatever registered a mod-

erate but significant rise in blood pressure (from an initial average of 119 mm Hg to an average of 129 mm). By the end of the 2nd week, however, the pressures had fallen to a point (average, 114 mm) slightly below the initial level. When the diet was fortified by the addition of thiamin at this point, there was an immediate rise in pressure, which reached an average of 142 mm after 2 weeks. By the end of the 5th week, although the pressures were still significantly high (138 mm), the general condition of the animals was poor, they had begun to show gross evidences of riboflavin deficiency, they had lost weight, and 6 of the original 50 animals had died.

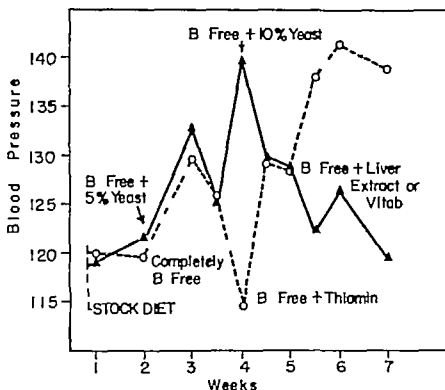


FIG 1 The effects of deficiencies of various vitamin B fractions on the blood pressure of rats (Experiment 1). All readings represent mean pressures which are lower than the actual systolic levels.

The second group of animals was intended to serve as controls. However, it was found that the addition of 5 per cent yeast was not sufficient to prevent a rise of blood pressure, for at the end of the 2nd week the average pressure had risen from an initial level of about 120 mm to 139 mm. Hg. Increasing the yeast supplement to 10 per cent was followed by a significant drop in pressure (to an average of 129 mm.) though not to normal levels during the 1 week of treatment. The further addition of liver extract or extract of rice polishings was followed by prompt return of the pressures to the original levels (average, 119 mm.) Since the effect of these two supplements was identical, the results have been pooled on the accompanying charts.

Fig 2 is a distribution curve of the pressure readings in these two groups of

HYPERTENSION CAUSED BY MALNUTRITION

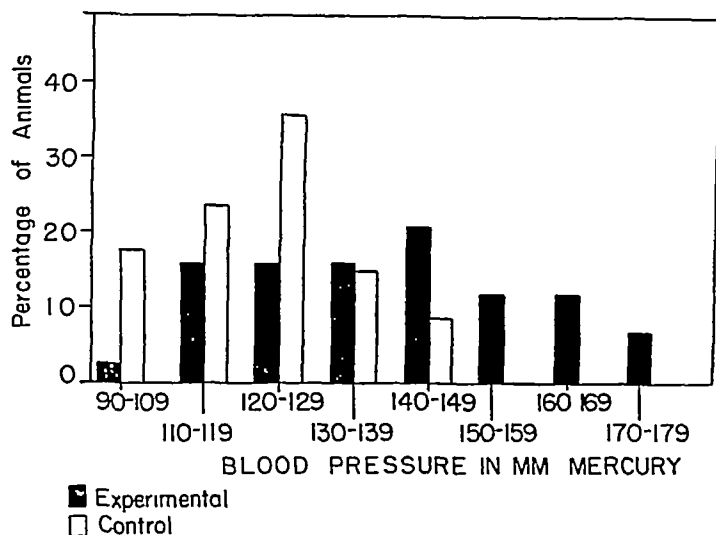


FIG 2 Distribution curves of the blood pressure readings at the conclusion of Experiment 1 (Fig 1) Hollow blocks represent well nourished group, solid blocks represent deficient group

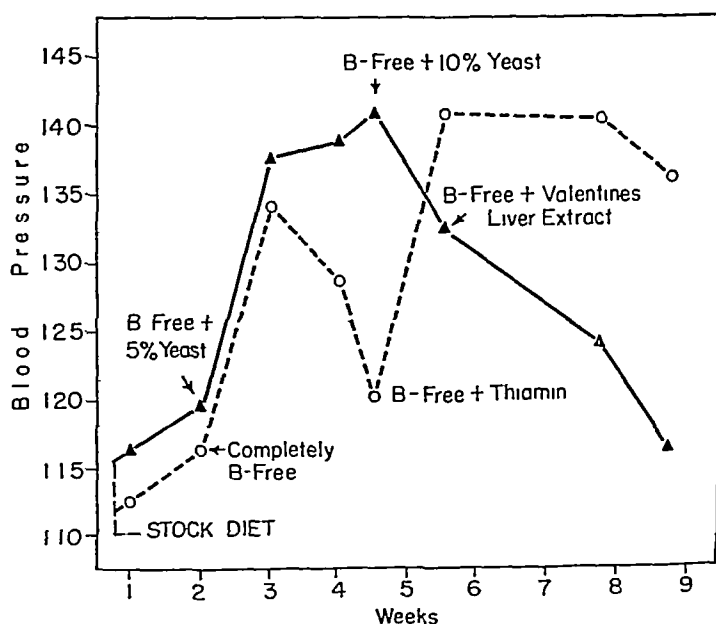


FIG 3 The results of Experiment 2, the design of which was almost identical with that of Experiment 1 (Fig 1)

animals at the end of the 5th week on the experimental diets. Fisher's *t* test shows that the difference in the blood pressures of the two groups was highly significant at the conclusion of the experiment ($P < 0.001$)

Experiment 2—This experiment was identical in design with Experiment 1, except for the facts that it was performed in summer rather than winter and that the rats were of a piebald strain. Each of the two groups consisted of 50 animals.

The results of this experiment, which are shown in Fig. 3, closely parallel those obtained in Experiment 1. The difference in the blood pressures of the two groups was likewise highly significant at the end of the experiment ($P < 0.001$)

Influence of a Partial Deficiency of the Vitamin B₂ Complex on Blood Pressure

Vitamin deficiencies of the completeness represented by the first two experiments seldom occur under ordinary conditions of living. On the other hand, partial deficiencies, particularly of the vitamin B₂ complex, are common. Experiment 3 was therefore designed to determine the effect of such partial deficiencies on the blood pressure.

Experiment 3—Five groups of 25 rats each were placed on the basic vitamin B free diet, plus thiamin by dropper. One group received liver extract (controls), a second group received no supplement whatever, a third group received autoclaved yeast in the amount of 2.5 per cent of the diet by weight, a fourth group received a similar supplement of 5 per cent yeast, and a fifth group was permitted to eat yeast as desired.

The results of this experiment are shown in Fig. 4. The group receiving liver extract maintained consistently normal pressures throughout the experiment (average, 118.4 mm Hg,⁷ standard deviation of the mean, 0.676). The group receiving no vitamin B₂ complex whatever showed a consistently elevated pressure throughout (average, 135.4 mm.,⁷ standard deviation of the mean, 0.844). The group receiving 2.5 per cent yeast likewise showed a consistently elevated pressure throughout (average, 142.2 mm.⁷ standard deviation of the mean, 0.898). The group receiving 5 per cent yeast also showed an elevated pressure, fluctuating between 135 and 140 mm.⁸ The

⁷ Snedecor's *F* test (24) shows that the variations of the weekly averages are no greater than would be expected from the variations of the individual pressures making up the averages. The average of the whole group is therefore used.

⁸ The *F* test shows that the variations of the weekly averages are slightly greater than would be expected from the variations of the individual pressures making up the averages. Pooling the individual readings and striking an average of the whole group is therefore unjustified.

group which was allowed to eat autoclaved yeast as desired, while theoretically provided with an adequate source of the vitamin B₂ complex, refused to eat the fortified diet during the early weeks of the experiment, and during this time their pressure rose to an average of 139 mm, gradually, however, as they ate the yeast better, their pressures dropped and at the end of the experiment the average was 124.5⁹

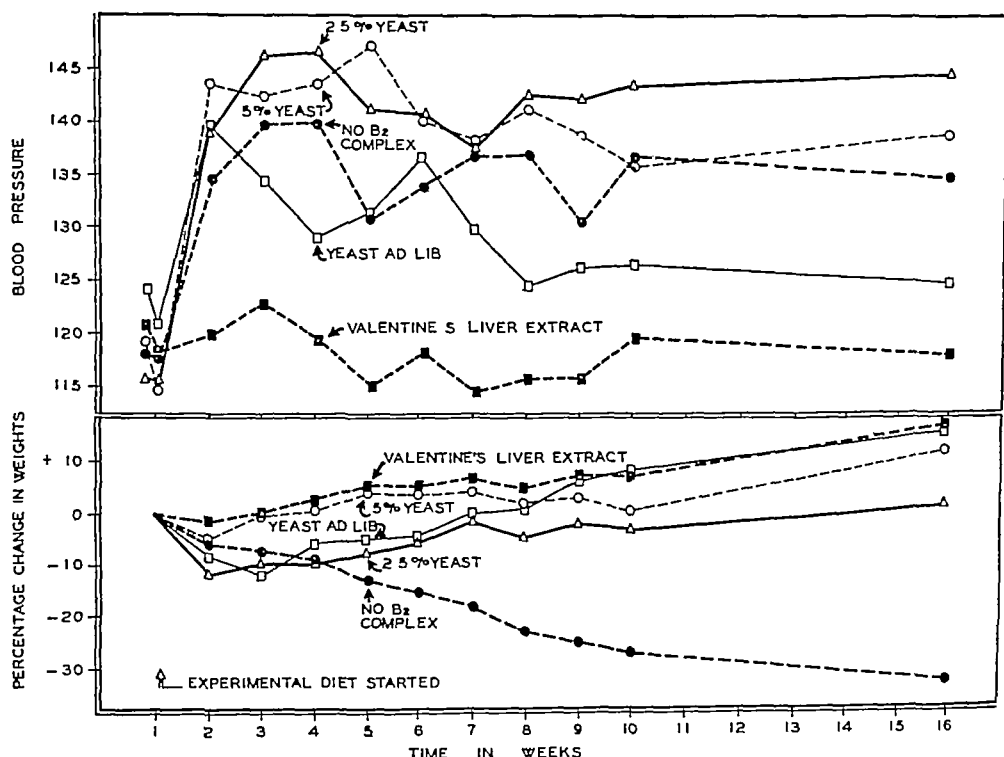


FIG 4 The effects of partial deficiencies of the vitamin B₂ complex on the blood pressure and weights of rats (Experiment 3)

Fisher's *t* test shows that the differences in pressures of the groups receiving no vitamin B₂ complex, as well as those receiving 2.5 per cent yeast, are highly significant when compared with the controls ($P < 0.001$). Although the weekly averages cannot be pooled in the case of the 5 per cent yeast group, the *t* test shows a highly significant difference between each weekly average and the corresponding control ($P < 0.001$). The weekly averages of the

⁹ The *F* test shows that the variations of the weekly averages are considerably greater than would be expected from the variations of the individual pressures making up the averages. Striking an average for the whole group is therefore unjustified.

group receiving yeast *ad libitum* also cannot be pooled. However, according to the *t* test, the differences between this latter and the control group are highly significant during the first 9 weeks of the experiment, but by the 10th week the pressures of this group have begun to fall closer to those of the controls (P , 0.02–0.01), and by the end of the experimental period there is no significant difference (P , 0.05–0.02).

Correlation of Changes in Blood Pressure and Weight—A comparison of the blood pressure readings with gain or loss of weight, as indicated in Fig. 4, shows that the blood pressure changes were not related to the general nutritional status of the various animals. For example, the group receiving 5 per cent yeast gained a total of 10 per cent in weight, the group receiving no B_2 complex lost 33 per cent of their weight, and yet the blood pressures of both groups rose to approximately the same levels during the course of the experiment.

Influence of General Undernourishment and Consequent Secondary Vitamin Deficiencies on Blood Pressure

It is generally agreed that the functions of many of the various accessory food factors are so closely interrelated that ascribing a given effect to deficiency of a specific dietary factor or even group of factors is a difficult matter. The part played by secondary vitamin deficiencies in inhibiting intestinal absorption has been especially emphasized by Susan G. Smith (20). She was able to show, for example, that when dogs are maintained on a black tongue-producing diet, they develop black tongue despite concurrent administration of nicotinic acid by mouth; if, however, the nicotinic acid be administered intravenously, the dogs are promptly cured. Obviously, therefore, the black tongue-producing diet causes its characteristic effects not simply because of its inadequate content of pellagra preventive factor but also because it leads to faulty utilization of this material, due apparently to poor intestinal absorption.

A similar situation has been shown to obtain in the case of vitamin A, for Underhill and Mendel (21) produced typical black tongue by means of a diet which Smith, Persons, and Harvey (22) showed to be deficient in vitamin A. Persons and Brown (23) were able to cure the black tongue so produced by the administration of nicotinic acid, huge doses were required by mouth, but small doses sufficed if given parenterally. Presumably, therefore, a deficiency of vitamin A produces black tongue by interfering with intestinal absorption.

Because of observations of this kind in nutritional studies, it was thought advisable to determine whether similar overlapping of vitamin functions obtained in the case of the dietary components under investigation in this study.

Experiment 4—Ten pairs of rats of identical weight were selected. One group was placed on the usual vitamin B-free diet supplemented by thiamin. The other group was similarly treated but in addition received liver extract in the amount of 1 ml per rat per day. The diets of this latter group were carefully weighed, and the total food consumption of each member was restricted to the amount consumed by his paired mate in the first group.

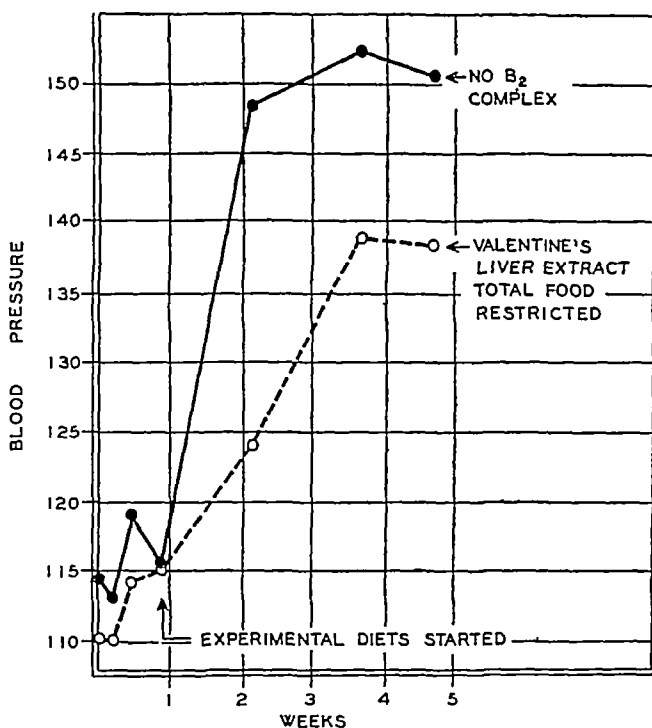


FIG 5 The effects of restriction of total food intake and consequent secondary nutritional deficiencies on the blood pressure of rats (*Experiment 4*). Note that the rise under such conditions is slower and less marked than when the rats are deprived entirely of the vitamin B₂ complex.

The weights of the two groups remained practically constant throughout the course of the experiment. The effects on blood pressure are shown in Fig 5. The rats receiving no vitamin B₂ complex showed the prompt and sustained rise in blood pressure seen in earlier experiments (from an initial average of 116 mm to approximately 150 mm). Those receiving a plentiful supply of liver extract but whose total food intake was restricted likewise experienced a rise in blood pressure, though the rise was much slower and less marked than in the former group (from an initial average of 110–115 mm to 138 mm).

As stressed by Susan G. Smith (20), relative speed and intensity of effect are important in deciding whether a vitamin is acting in a primary or secondary capacity. On the basis of these criteria, it would appear that the rapid and high rise of pressures seen in the group receiving no vitamin B₂ complex indicates that this complex is acting in a primary capacity, whereas the slower and less marked rise noted in the other group may be the result of secondary nutritional deficiencies.

A Check on the Reliability of the Indirect Method of Determining Blood Pressure

The indirect method of determining blood pressures in the foregoing experiments is subject to question because the preliminary heating introduces

TABLE I
Maximal Blood Pressure of Deficient and Well Nourished Rats As Determined by Insertion of a Cannula into the Abdominal Aorta

Blood pressures of deficient rats		Blood pressures of well nourished rats	
	mm. Hg		mm. Hg
	155		135
	135		128
	135		125
	140		125
	155		110
	135		110
	165		120
	130		115
			115
Average	143.75		120.33

df 15 *t* 4.4938 *P* < 0.001

an abnormal variable. Although this variable was minimized by heating all animals for identical periods of time and was thus a reduplicated error, it seemed advisable to check the accuracy of the indirect method by direct measurements of the pressure in the cannulated abdominal aorta.

Experiment 5—Two groups of 10 rats each were placed on the usual vitamin B-free diet supplemented by thiamin. The first group received no other supplement, but the second group was given liver extract. After having been on these diets for 5 weeks the animals were anesthetized by the intraperitoneal injection of nembutal. The abdomen was then opened and a cannula inserted into the abdominal aorta. A solution of heparin was injected to prevent clotting and the cannula was connected with a mercury manometer.

The results of this experiment are detailed in Table I, in which are recorded the maximal pressures (mean) attained by the animals which survived the

procedure The average maximal pressure of the deficient group was 143.8 mm Hg (standard deviation, 12.75), while the average maximal pressure of the well nourished group was 120.3 mm Hg (standard deviation, 8.57). These differences are of the same magnitude as those obtained by the indirect method used in the preceding experiments. The accuracy of the indirect method is therefore attested.

It was noted incidentally in the course of this experiment that the deficient animals were very intolerant of ether. A few whiffs of this agent, administered for the purpose of deepening the anesthetic effect of nembutal, were sufficient to cause immediate death. It was also observed that when the cannula was first inserted, the blood pressures of the deficient animals tended to be low and to rise gradually as the observations were continued for the ensuing 30 or 40 minutes. In contrast, the pressures of the well nourished group remained relatively constant throughout the period of observation. Presumably, therefore, the deficient animals were much more subject to shock than were the well fed rats.

DISCUSSION

The results of the above experiments indicate that a deficiency of the entire vitamin B complex is followed by a slight fall in the blood pressure of rats. A deficiency of only the heat-stable fractions, on the other hand, is followed by a significant and persistent rise in blood pressure, which can be combated successfully by restoring these factors to the diet. Partial deficiencies are even more effective than are complete deficiencies, probably because of the marked debilitating effects of the latter.

These studies also show that even an excessive intake of the vitamin B₂ complex does not protect the animals completely if the diet is otherwise deficient. It is known from studies on other deficiency states, such as canine black tongue, that various non-specific nutritional inadequacies may often duplicate the pathologic picture of a specific deficiency. There is good evidence that these non-specific nutritional factors exert their effects by interfering with the absorption and utilization of the specific vitamins. It is possible, therefore, that an analogous mechanism may be responsible for the rise of pressure seen in those animals whose total food intake was restricted.

At the present time, knowledge of the functions of the vitamin B complex is so incomplete that it is impossible accurately to define the mechanism whereby these deficiencies mediate a rise in blood pressure. One theoretical possibility is that some types of hypertension may be metabolic in origin, the crucial defect being incomplete oxidation in the kidney as a result of an inadequate supply of respiratory enzymes. Some components of these enzyme systems are an integral part of the structure of the cells themselves, others, such as the prosthetic groups of coenzymes I and II and the flavins,

have to come from exogenous sources. If the intake of these latter components be restricted by dietary manipulation, one would expect that the integrity of those systems to which they contribute would be impaired. As a consequence, the oxidative processes which depend on those systems would be retarded. The disturbance produced in this manner would, of course, be a generalized one, in which the kidney would share. The effect on this organ would be a slowing of the rate at which oxygen is consumed, with resultant impairment of its normal metabolic functions. This is the same physiologic abnormality as that produced by constriction of the renal arteries, though the two mechanisms are obviously different. This idea is advanced not as the proven explanation of the blood pressure rise seen in the present studies, but as a working hypothesis for further enquiries into the etiology and pathogenesis of the hypertensive states.

SUMMARY

The effects of dietary deficiencies on the blood pressure of rats were studied, with especial reference to vitamin B deficiencies. A deficiency of the entire vitamin B complex was followed by a slight fall in blood pressure. A deficiency of only the heat-stable fractions was followed by a significant and persistent rise in pressure, which could be reversed by restoring these factors to the diet. Partial deficiencies were followed by a higher rise of blood pressure than were complete deficiencies, perhaps because of the debilitating effects of the latter.

Even an excessive intake of the heat-stable fractions of the vitamin B complex did not prevent entirely a rise of pressure if the diet was otherwise deficient. Under the latter conditions, the rise in pressure was slower and less marked than in those animals with a deficiency of the vitamin B₂ complex only. It therefore appears that, while a deficiency of the vitamin B₂ complex plays a dominant rôle in causing a rise of blood pressure in rats, other dietary factors as yet undefined are also involved. From analogy with other deficiency states, it is possible that these undefined nutritional factors cause their effects by interfering with absorption and utilization of the vitamin B₂ complex.

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RADIOACTIVE IRON ABSORPTION IN CLINICAL CONDITIONS NORMAL, PREGNANCY, ANEMIA, AND HEMOCHROMATOSIS*†

By W M BALFOUR, M D., P F HAHN Ph.D., W F BALE, Ph.D.,
W T POMMERENKE, M D., AND G H WHIPPLE, M.D

(From the Departments of Pathology, Radiology, Obstetrics and Gynecology of the
University of Rochester School of Medicine and Dentistry Rochester, New York)

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The experiments listed below suggest very strongly that when the *iron reserves are depleted* the human being will absorb iron in relative abundance—in fact 10 to 20 times normal. Although the iron stores of the body are mainly in liver, spleen, and bone marrow, these stores influence iron absorption in some obscure fashion. The iron content of the *mucosa of the gastro-intestinal tract* is very low (2) and there appears to be little if any iron storage here, but the cells of the mucosa do have the power to accept or refuse iron—a reaction totally different from that relating to most metals or salts. Low hemoglobin levels in the blood (anemia) are not enough to call out active iron absorption if the reserve stores are abundant (6). At the moment an adequate explanation of the observed facts is not at hand but continued study of the mucosa of the gastro-intestinal tract is obviously indicated.

For some good reason the body guards itself against too liberal an intake of iron, perhaps because it finds the elimination of iron difficult. There is some evidence that a considerable excess of iron will harm those cells in which the iron is stored—for example the liver and pancreas cells in hemochromatosis. The older clinical belief that iron was absorbed and the surplus removed by excretion through the mucosa of the large intestine must be put aside even though this belief has the prestige and academic tenure of many decades of acceptance by physician and physiologist. Some of this misconception was due to iron balance studies in which too much reliance should not be placed. Difficulties in iron analyses are considerable, particularly of feces, where phosphorus-iron compounds introduce serious errors and make the figures for iron recovery too low, thus giving an erroneous impression of positive iron balance.

Welch, Wakefield and Adams (15) by means of an ileostomy in a single patient showed that the *excretion* of iron into the colon was negligible in

* We are indebted to Eli Lilly and Company for aid in conducting this work.

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amount McCance and Widdowson (10, 11) by parenterally introduced iron showed that the human body could *eliminate* iron only in small amounts. Their general conclusions were in harmony with our belief that *absorption* controls iron balance and *reserve stores* in some way control absorption.

Radio iron is perfectly adapted to the study of the elusive iron element as it shuttles about within the body. Probably by no other means can we get a comprehensive understanding of the true internal iron metabolism. By use of radio iron in dogs we were able to bring convincing evidence (5) that the plethoric dog (with ample reserve stores) would absorb little or no radio iron, that the normal dog (hematocrit 50 per cent red cells) would absorb little radio iron, but the depleted anemic dog would absorb much (5 to 50 per cent) of the ingested radio iron which would promptly appear in new red cells in the circulation. Furthermore dogs, anemic or normal, can eliminate very little iron and that largely by way of the liver and bile (4).

Dogs are ideal experimental animals for the study of iron metabolism and we know a good deal about their capacity to form new hemoglobin under the stimulus of anemia. We believe their gastro-intestinal tract behaves toward iron much as does the human being. The experiments below support this claim. It is possible to do preliminary experiments in the dog with much better control than is possible with the human being but eventually we must always make similar, if incomplete, observations with human beings to exclude possible differences between the physiology of the dog and man. Furthermore, we can study diseased states in the human patient which have not been reproduced in the dog—for example, pernicious anemia and hemochromatosis.

As has been pointed out before (4), when iron is made *radioactive* by bombardment in the cyclotron, only an infinitesimal portion of the iron atoms is changed from the normal Fe^{56} isotope to the unstable and therefore radioactive isotope Fe^{59} . Every atom of iron that emits a beta ray detected by the Geiger counter has been during its whole previous history since bombardment in the cyclotron, an atom of iron differing from ordinary iron only by its small increase in atomic weight. Since there is abundant evidence to indicate that the animal organism cannot differentiate significantly between atoms with these small variations in mass, we can have every confidence that the path of these radioactive atoms that we can follow with the Geiger counter represents in every detail the pathway of the total amount of administered iron tagged by the radioactive iron isotope. Subsequently in this paper we refer to iron tagged in this manner as *radio iron*.

Experimental Methods

The radioactive isotope of iron (47 day half-life) used in these experiments was prepared in the cyclotron of the Radiation Laboratory of the University of California, using the methods described by Wilson and Kamen (16). Before feeding, it was

usually further purified and freed from the last radioactive contamination by repeated ethyl ether extraction of the chloride adding neutral manganese and cobalt salts in excess as carriers. It was then made up as ferric ammonium citrate, in which form it was fed.

Radioactive measurements before January, 1941, were made, using the dipping counter technique described in an earlier paper (1, 5). Data obtained since that time were obtained by use of much more sensitive detection equipment. The other experimental techniques involved in blood studies, isolation of radio iron electroplating, etc., have been described in earlier papers (3).

Direct determination of red cell circulating mass was not practicable in these cases. Therefore, the estimate of red cell mass was made as follows. By plasma dye dilution methods there is considered to be about 80 ml. of blood per kilo of body weight. However, it has been pointed out that the red cell volume as calculated from the plasma volume and venous hematocrit is about 25 per cent too high in dogs (7, 9) and this has been reported to be true also in human beings (14). Therefore the red cell mass was estimated as follows:

$$\text{Body weight in kilos} \times 80 \times \text{venous hematocrit per cent} \times 0.75 = \text{cell mass}$$

We have expressly included in our tables values for per cent of the isotope fed per 100 ml. of red cells so in the event that other estimations of cell mass may prove more accurate, the total radio iron in circulation may be calculated on the latter basis. The circulating isotope in the tables is calculated by multiplying the concentration of isotope in red cells by the estimated mass of red cells.

The patients marked "*" in the following protocols received labelled iron at a time when our measuring equipment was 30 times less sensitive than that now used. Although the dosage activity was quite high in most of them, the amount of isotope appearing in the red cells was very near the limits of error of the methods as used at that time. Therefore it is not possible to estimate accurately the amount of radio iron in the circulation. In each instance the figure given represents an *upper limit* which is reached by assuming a conservative count which could have been detected and calculating the amount which would have been present if this count had actually been found.

Clinical Histories¹

Case 1 B G (Hospital No 182641) Male, age 57 *Peptic ulcer with hemorrhage*. The patient had ulcer symptoms for about 1 year. They increased in severity during the week before admission, and during the last 4 days of this week the patient noted black stools. There were increasing weakness and dizziness and vomiting of "coffee-ground" material. Admission blood studies RBC 1,500,000 hemoglobin 4 gm. per cent. He was given small frequent transfusions and on the 4th hospital day 17 mg of radio iron as ferric ammonium citrate was given. His hemoglobin on

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this day was 61 gm per cent Stool on the day of feeding showed 1+ guaiac, but subsequent examinations were negative for blood He had been bleeding at least 10 days before the radio iron was given and probably longer Neutral iron was begun 4 days after the radio iron was given Blood samples were obtained at 5, 8, 11, 17, and 24 days following the feeding and activity measurements were made on the separated red cells The 11 day cells showed 1.48 per cent of the fed iron per 100 ml of red cells An estimate of the patient's circulating red cell mass was 1005 ml Thus, it was calculated that 15 per cent of the administered dose was present in the circulation at this time This patient was sampled a number of times over the course of 45 days

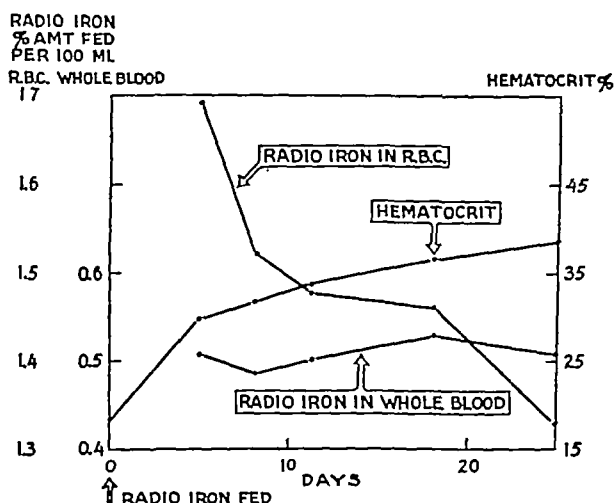


CHART A Bleeding gastric ulcer Case 1, Table 1, B G

In Chart A are plotted the concentrations of radio iron per 100 ml of the red cells of case 1, Table 1, the concentrations per 100 ml of whole blood and the red blood cell hematocrit readings for the corresponding values. One notes that over the whole experimental period the concentration of radio iron per 100 ml whole blood remains constant. During this time the hematocrit had risen from about 19 per cent to 37 per cent. At the same time there had been a progressive decrease in the red cell isotope concentration. If one assumes that the total circulating blood volume of this patient remained constant over this period, an assumption probably valid in the light of other recently published data (3), this result would indicate that at least all of the absorbed radio iron which was going to be utilized to form hemoglobin had been so used as early as the 5th day after feeding. Since there was a need for new hemoglobin and in view of the fact that hematopoiesis was progressing rapidly at this time, it would be a fair inference that the figure of 15 per cent utilization also represented the major portion of the radio iron absorbed from the gastro-intestinal tract.

Case 2 V P (Hospital No 80540) Male, age 27 *Duodenal ulcer with hemorrhage* A third admission to this hospital with severe hemorrhage from the duodenal ulcer Other admissions were 8 and 3 years previously Following each he remained on iron and diet therapy for short periods. Short follow-ups revealed a hemoglobin remaining around 12 gm. per cent The third hemorrhage began after long continued dietary indiscretions. Symptoms of bleeding apparently began 6 days before admission. The red cell count on admission was 1 600 000, and the hemoglobin 6 gm. per cent. He was treated with bed rest and progressive Sippy diet, and the hemorrhage stopped after one hospital day Two small transfusions were given On the 14th hospital day, when his red count was 3 800 000 and his hemoglobin 10.2 gm. per cent, he was given a single feeding of 20 mg. of radio iron as ferric ammonium citrate. 7 days later the red cells contained 1.85 per cent of the administered dose per 100 ml. This corresponded to an estimated 20.2 per cent of the fed dose in circulation 46 days after feeding, the hematocrit had risen to 47 per cent and the red cell isotope amounted to 1.42 per cent of the amount fed. The amount estimated in circulation was essentially the same (20.6 per cent) due to the increased mass of red cells (3)

Case 3 A. D * (Hospital No 127050) Female, age 22 para 2, gravida 4. *Incomplete abortion* Last menstrual period 2 months previously Slight bleeding began 2 weeks before and *profuse bleeding* with passage of clots 4 days before admission Admission blood studies red blood cells 2 280 000, hemoglobin 5 gm. per cent. On the 2nd hospital day she was given 200 cc. of whole blood Because of repeated chills and fever the abortion was not completed until the 17th hospital day 10 days before curettage and 3 weeks after the bleeding began she was given by mouth 423 mg. of radio iron as ferric ammonium citrate. At this time the red count was 3 000 000 and hemoglobin 6.8 gm. per cent. 4 days later a blood sample revealed that the red cell radio iron was 0.2 per cent of the dose fed per 100 ml. of red cells, or an estimated 1.3 per cent of the dose in circulation.

Cases 1, 2, and 3 (Table 1) may be considered together and show an iron absorption many times normal. With *severe anemia* due to bleeding from a duodenal ulcer (case 2) there had been previous episodes of bleeding and probably much of the *iron reserve* had been exhausted For this amount of ingested iron (18 to 20 mg. Fe) we may say that an expected normal absorption would be 0.5 to 1.5 per cent (case 14) The absorption in these two cases is 15 and 21 per cent respectively—more than 10 times the expected absorption of a normal individual. The intestinal mucosa accepts this iron readily and the hemopoietic mechanism builds it very rapidly into hemoglobin within new red cells

Case 3, an incomplete *abortion* with bleeding and *anemia* 2 to 3 weeks before iron feeding, shows considerable iron absorption. The dose of radio iron was large (423 mg. Fe) because the radio activity was low In standard dogs with this dose we expect 5 to 7 per cent absorption and utilization and we could hardly expect more than this in a human In other words, this human absorption (1.3 per cent) is 20 to 25 per cent of maximal, or twice or three times an expected normal. It is probable that this bleeding had not decreased the

reserve stores of iron as completely as in cases 1 and 2. Moreover, the interval between feeding and sampling was only 4 days, which may not have allowed sufficient time for the total radio iron utilization and appearance as new hemoglobin (7).

Case 4 J H (Hospital No 163765) Male, age 11 months *Impetigo contagiosa*. This boy had had a series of infections of the skin which, because of poor environmental conditions, were difficult to control. Impetigo on this admission had been present about 2 months. Blood studies showed a red count of 4,700,000 and a hemoglobin of 11 gm per cent. There was no fever and little systemic reaction. It was thought that there might be a nutritional basis for the anemia, although not necessarily due to iron deficiency. 3 days after feeding 8 mg of radio iron as ferric ammonium citrate, 0.75 per cent of the administered iron was present per 100 ml. of red cells. 5 and 7 days after feeding, the amounts present were 1.21 per cent and 0.98 per cent per 100 ml in the cells. The amount present in the circulation as determined by concentration in 7 day cells was 2.2 per cent of the amount fed.

Case 5 H K* (Hospital No 172905) Male, age 21. Chronic *pyelonephritis* with severe secondary anemia. This patient had been sick for several months. On admission the red count was 1,600,000 and the hemoglobin 8.5 gm per cent. The urine contained albumin and white cells with no red cells. The N P N was 180 mg per cent. Iron and a long trial of intramuscular liver had had little effect on the anemia. A single dose of 42 mg of radio iron was given as ferric ammonium citrate. 7 days later there was no detectable activity in the red cells. Because of the counter used and the low activity of the sample given, it is possible that the maximum absorption in this case was 3.7 per cent. However, it is highly probable that the absorption was less than 1 per cent.

Case 6 L S* (Hospital No 167067) Female, age 44. *Hypochromic anemia* with gastric anacidity. The history dates back about 10 years. During this period she had been treated elsewhere for anemia by various oral liver products with little effect. Symptoms recently increased. She noted difficulty in swallowing and a tendency toward spoon-shaped nails. Admission blood studies: red count 4,200,000, hemoglobin 7.5 gm per cent. No free gastric HCl after histamine. She was given a single dose of 204 mg of radio iron and 6 days later activity measurements of the red cells showed a very small count which would represent a maximum of about 2.0 per cent of the amount fed in circulation. On a régime of ferrous sulfate and HCl, her symptoms gradually decreased over a period of 2 months. During this time her hemoglobin rose to 13.7 gm per cent with a slight increase in the count.

Cases 4, 5, and 6 (Table 1) are considered together—anemias in which there should be some absorption but a relatively insignificant response is recorded. Case 4, *impetigo*, and case 5, *pyelonephritis*, show a utilization perhaps not in excess of normal. It should be noted that the degree of anemia in case 4, *impetigo*, is only slight or close to a low normal hemoglobin. It has been shown in standard anemic dogs that an *infection* will not prevent absorption but will delay the utilization of the hemoglobin building material (13). So it is pos-

sible that some iron was absorbed but not utilized in the 7 day period between feeding and sampling for the radio iron

Case 6 is an interesting type of *hypochromic anemia* which had been under treatment at various times Lack of gastric HCl may have been a factor She did absorb somewhat more than normal (2 per cent) of a 204 mg dose of radio iron The maximum anemic absorption would be 5 to 10 per cent and the normal absorption less than 1 per cent Therapy of ferrous sulfate and HCl did bring her blood back to normal within 2 months

TABLE 1
Radioactive Iron Uptake by Red Cells in Various Clinical Conditions

Case	Diagnosis	Weight	Estimated red cell mass	Hematocrit at feeding	Hematocrit at sampling	Interval between feeding and sampling	Dose iron	Per cent amount fed per 100 ml. red cells	Per cent amount fed circulating estimated
		kg	ml.	per cent	per cent	days	mg	per cent	per cent
1 B G	Gastric ulcer—hemorrhage	49.4	1005	18.4	33.8	11	18	1.48	15
2. V P	Duodenal ulcer—hemorrhage	51.8	1455	29.4	46.7	7	20	1.37	20.2
3 A. D.*	Abortion, hemorrhage	50.0	630	22.1	20.8	4	423	0.20	1.3
4. J H	Impetigo	9.8	220	35.3	37.9	7	8	0.98	2.2
5 H. K.*	Chronic pyelonephritis	52.6	600	18.4	19.0	7	42		0.3
6 L. S.*	Hypochromic anemia	64.6	1150	32.0	29.4	6	204	0.17	2.0
7 A H	Pernicious anemia	60.0	1090	23.4	30.3	10	20	0.12	1.3
8 S. M.*	Leukemia	8.9	130	24.5	—	6	125	0.47	0.6
9 J V*	Familial icterus	11.8	215	—	30.5	7	93	0.13	0.3
10 A. Z.*	Mediterranean anemia	14.3	46	6.6	5.3	4	15		0.15
11 C. Y.*		13.0	175	23.2	22.7	5	64	0.26	0.5
12 H V	Hemachromatosis	61.2	1610	45.6	43.8	6	53	0.04	0.7
12.		61.2	1610	41.0	43.4	7	14		0.8
13 S V	Polycythemia	59.0	1750	—	49.6	6	204	0.03	0.5
14. A. K.	Normal	81.0	2260	48.5	46.6	7	20	0.08	1.8

Case 7 A. H (Hospital No 184793) Male, age 64 Untreated *pernicious anemia* Symptoms had been gradually increasing over a period of 4 months. Recently evidence of spinal cord damage had appeared. On admission, the patient was almost moribund Blood studies red blood cells 1,300,000 hemoglobin 5.9 gm. per cent, white blood cells 2,450 Bone marrow smear was typical of Addisonian type of macrocytic anemia Two doses of intramuscular liver had been given when he received 20 mg of radio iron as ferric ammonium citrate. At the time of feeding the red cell hematocrit was 23.4 per cent A considerable hematopoietic effect was induced by continued intramuscular liver and 18 days later the hematocrit was 38 per cent 10 days after feeding the isotope the red cell radio iron was 0.115 per cent of

the administered dose per 100 ml of red cells corresponding to an estimated 1.3 per cent of the dose in circulation. No therapeutic iron was given during the course of these observations. He was discharged on the 26th hospital day with normal blood studies and evidence of considerable improvement in the cord changes.

Case 7 (Table 1) is of particular interest. A typical case of *pernicious anemia* rapidly producing new hemoglobin due to the liver therapy would presumably absorb iron rapidly. The iron absorption (1.3 per cent) is not more than that of a normal control (1.8 per cent) given the same dose of radio iron. Obviously the iron needed for new red cell and hemoglobin production (case 7) comes from iron *reserve stores* which are well known to be much above normal in untreated pernicious anemia. We believe that the presence of these reserve stores of iron in some fashion inhibits iron absorption which takes place so readily in simple anemia due to blood loss when the reserve stores are depleted.

Case 8 S M* (Hospital No 168514) Female, age 7 months *Leukemia*, probably monocytic (autopsy). This Italian baby had several admissions because of *severe anemia*. Splenomegaly and hepatomegaly were marked. The diagnosis of Mediterranean anemia (Cooley's type) was considered. The leucocyte count was low-normal with a high percentage of lymphocytes. During one admission she was given 125 mg of radio iron in the form of ferric ammonium citrate. Red cells taken 2 hours after feeding showed no radio iron. Cells taken 6 days after administration showed 0.47 per cent of the amount fed per 100 ml of red cells, or about 0.6 per cent of the amount fed in circulation. No more blood samples were obtained. Her red count at the time of feeding was 3,140,000 and hemoglobin 8.2 gm per cent. She was admitted again 4 months later in poor condition with a very severe anemia and bronchopneumonia. At autopsy specimens of the *liver*, *spleen*, and heart were obtained. The 15 gm liver sample contained an amount of radio activity which would represent about 0.83 per cent of the administered dose in the whole 530 gm liver. A 26 gm aliquot of the 1100 gm spleen indicated a radio iron content in this organ of 0.56 per cent of the administered dose. The heart, by similar analysis, contained about 0.09 per cent. Thus, 4 months after feeding radio iron 1.5 per cent of the dose given was present in these unperfused organs.

Case 8 (Table 1) supplies us with data on autopsy material in *leukemia*. The patient was given radio iron and 6 days later showed 0.6 per cent in the circulation, but at *autopsy* 4 months later the liver and spleen contained 1.5 per cent of the dose fed 4 months previously. Obviously 1 per cent of the dose of radio iron (or more) was absorbed but not incorporated in new red cells within 6 days in spite of the anemia. The severe anemia of leukemia is probably due to choking of the red marrow with abnormal white cells thus impairing red cell production—a blockade of the hematopoietic process. This case with severe anemia did absorb about four times normal (or more) but did not utilize the radio iron to make hemoglobin, perhaps due to an abnormal marrow.

Case 9 J V* (Hospital No 170116) Male, age 2 years Familial *hemolytic icterus* This patient was admitted with symptoms of a severe anemia without jaundice. Blood studies revealed small cells with increased fragility and tendency toward spherocytosis. Similar changes were found in maternal blood 93 mg of labelled iron was fed as ferric ammonium citrate in milk. His red count at this time was 2,000,000 and hemoglobin 4.6 gm. per cent. 7 days later the activity in the red cells was extremely low, but the amount of isotope in circulation could be placed at a maximum of 0.3 per cent of the amount fed. Following transfusions and general supportive measures, splenectomy was performed with complete relief of symptoms.

Case 10 A Z* (Hospital No 121099) Female, age 4 years *Erythroblastic anemia* This Italian girl had had the diagnosis made at 14 months of age. A brother had died of the same disease. During 19 hospital admissions there was a history of repeated intercurrent infections and she had been transfused many times. Red cell count was 0.830,000 and hemoglobin 2 gm. per cent at the time a single dose of radio iron of 15 mg was given as ferric citrate. 4 days later the red cell activity was very near the background count, but it was estimated that less than 1.7 per cent of the material fed was in circulation.

Case 11 C Y* (Hospital No 128287) Female, age 3½ years *Erythroblastic anemia* At the time of feeding the red count was 2,800,000 and the hemoglobin 7.9 gm. per cent. 532 nucleated red cells per 100 white cells were counted. She was given 64 mg of radio iron in a single dose as ferric citrate. 5 days later the red cell activity was extremely low, and the maximum amount of the isotope estimated to be in circulation was about 0.45 per cent of the dose fed.

Cases 9, 10, 11, and 12 (Table 1) *Familial icterus, Mediterranean anemia* (Cooley), and *hemochromatosis* have several important abnormalities in common and some striking differences. All three conditions show *maximal* figures for *iron storage*, 10 to 20 or more times normal in the liver for example. Iron absorption in all three conditions is *minimal* in spite of severe anemia in the children with Mediterranean anemia and familial icterus. There was no anemia in hemochromatosis but bleeding was instituted to deplete the reserve iron stores if possible. Anemia due to blood loss was produced but there was still no significant iron absorption. Again it appears that the presence of *abundant iron stores* in the body in some way inhibits iron absorption. Obviously at some time, probably *early* in these chronic disease conditions, iron was absorbed in abundance.

Case 12 H. V (Hospital No 138938) Male, age 49 *Hemochromatosis* History dates back about 5 years with first symptom being increasing pigmentation of the skin. This was followed by the onset of diabetes mellitus and finally by distention of the abdomen due to fluid. On the first admission, the diagnosis was confirmed by skin biopsy which showed hemosiderin granules and iron-staining pigment about the sweat glands. Infra red photography revealed extensive collateral circulation in the abdominal wall. There were pronounced muscular weakness and evidence of myocardial damage. Loss of hair, impotence, decrease in testicular size

and a low basal metabolic rate (-43 per cent) indicated involvement of other endocrine glands. The diabetes was well controlled by diet and 60 units of protamine insulin per day. On a subsequent admission a single dose of 60 mg of radio iron was given by mouth as ferric ammonium citrate. Samples were taken at intervals for 3 hours after feeding to determine whether any absorption had occurred as indicated by presence of the isotope in the plasma. Further sampling was done 1, 2, 5, and 6 days following administration, and the amount of radio iron expressed as per cent of the dose fed found in the red cells was 0.012 at the highest reading.

The blood picture in hemochromatosis is usually near normal, as it was in this case, and it might be argued that such a patient might absorb iron and not utilize it for hemoglobin formation, since there was no demand for increased hematopoiesis. Therefore, the patient was subjected to a phlebotomy amounting to 500 ml to provide a stimulus for red cell formation. 2 days after bleeding the isotope concentration in the red cells was 0.017 per cent of the amount fed. 10 weeks later there was 0.04 per cent of the amount fed per 100 ml of red cells. It was thought proper to determine whether intermittent heavy bleeding might be effective in marshalling deposited iron from the tissues for hemoglobin formation. If the hemosiderin were reversibly deposited, it might be expected that eventually the excess iron could be removed and possibly the fibrotic changes in the liver and endocrine glands arrested. If radio iron had been absorbed and deposited along with the other iron in the tissues, it might be possible to follow the removal of this iron. However, after each of several bleedings the red cell count in the patient dropped progressively, and even though the hematocrit and hemoglobin content of the blood remained near normal, it was felt advisable to suspend attempts at therapy at this time. Following the second bleeding, the patient was given 14 mg of radioactive iron, and even though his red count was about 3,800,000 subsequent sampling showed he had absorbed only 0.8 per cent of this small dose. Subsequent bleedings and samplings showed no rise in the red cell isotope concentration.

Case 13 S V* (Hospital No 167389) Male, age 42. Chronic bronchitis and bronchiolitis with secondary *polycythemia*. This patient had numerous phlebotomies with symptomatic relief, the last one several months before the iron was given. The red count was 6,500,000 and the hemoglobin 15 gm per cent at the time he was fed a single dose of 204 mg of radioactive iron and 6 days later the red cell activity was practically 0. The maximum estimated amount of the iron in circulation was 0.5 per cent of that fed. Two phlebotomies were performed following the feeding of the iron, and the red cell activity did not change.

Case 14 A K. (Hospital No 157144) Male, age 25. Normal. This medical student was fed radio iron as a normal control. Labelled iron was given in very low doses of 4 mg per day for 5 days since this was felt to favor maximum absorption. 7 days after the last feeding there was 0.08 per cent of the administered radio iron per 100 ml of red cells. This corresponded to an estimated 1.8 per cent of the dose.

in circulation. Since the blood picture was normal and there was presumably no stimulus for hematopoiesis over and above the replacement demands, 500 ml of blood was removed in order to provide the stimulus. 7 days later the red cells showed 0.09 per cent of the administered dose per 100 ml corresponding to an estimated 1.8 per cent of the dose in circulation.

Case 13 (Table 1) *Polycythemia* It might be suspected that the over production of red cells in this disease might be due to an abnormally high iron absorption. Here is definite evidence again that surplus reserves (even if in the circulation) inhibit iron absorption. This case shows only traces of radio iron in the circulating red cells, 6 days after feeding.

Case 14 (Table 1) A *normal control* was given very small amounts (4 mg Fe) of active samples of radio iron over 5 consecutive days to get a *maximal absorption*. In anemia due to blood loss with repeated similar small feedings one might expect 50 to 60 per cent utilization of iron. The maximum after 7 days in the normal case was 1.8 per cent. Subsequent bleeding to bring out of storage any unused absorbed radio iron showed a maximum figure of 1.8 per cent radio iron in the circulating red cells. Compared with cases 1 and 2 (Table 1) this represents not less than a 10 to 1 differential in favor of the human case of ulcer and anemia due to blood loss. Actually the differential was greater because the ulcer patients received larger single doses of iron which are not utilized so effectively as are the small doses.

Clinical Histories—Pregnancy

Case 21 M C (Hospital No 143507) Age 37 para 2 gravida 5. Pregnancy with hypertension and paroxysmal auricular tachycardia. Gestation 2 months, therapeutic abortion. Patient complained of vertigo, scotomata, headache and paroxysmal dyspnea. Heart was enlarged, with presystolic and systolic murmurs. Blood pressure was 220/115. At the time of iron feeding red cells were 4,200,000, hemoglobin 14.7 gm per cent, hematocrit 43.3. She was given 16 mg of radio iron and delivered by hysterotomy 1 week later. Subsequent sampling showed an estimated 2.7 per cent of the amount fed in circulation.

Case 22 P F (Hospital No 165477) Age 27 para 2. Pregnancy with rheumatic heart disease. Class II A. Gestation 3 months, therapeutic abortion. Pregnancy was subjectively uneventful except for dyspnea on exertion. Examination disclosed mitral and aortic stenosis and insufficiency. Blood pressure 140/42. Urine showed occasional red and white cells. At the time of iron feeding blood studies showed red count of 4,110,000, hemoglobin 14.1 gm per cent, hematocrit 37.5 per cent. She was given 53 mg of radio iron and pregnancy was interrupted 7 days later. Subsequent sampling revealed an estimated 4.9 per cent of the amount fed in circulation.

Case 23 J F (Hospital No 101086) Age 24 para 3 gravida 4. Pregnancy, abnormal multiple, with chronic nephritis and bilateral hydronephrosis. Gestation 4 months, therapeutic abortion. In her two previous pregnancies the patient developed

nephritic toxemia with chronic nephritis The first pregnancy was complicated by pre-eclampsia During the present pregnancy, she developed headaches, nausea, vomiting, ankle edema, and albuminuria Blood pressure was 125/90 Retrograde x-ray studies showed bilateral hydronephrosis At the time of feeding, the red count was 4,430,000, hemoglobin 14 gm per cent, hematocrit 43.7 per cent She was given 122 mg of radio iron and the pregnancy was interrupted 2 days later Sampling revealed 4.2 per cent of the amount fed appearing in the circulation

Case 24 E L (Hospital No 171540) Age 25 years, para 2, gravida 3 Pregnancy with nephritic toxemia, without convulsions Gestation 4½ months, therapeutic abortion 3 years previously she had had a pregnancy complicated by albuminuria During this pregnancy she had headaches, ankle edema, blood pressure 185/120, and albuminuria amounting to 0.5 gm per liter At the time of feeding, blood studies showed 4,000,000 red cells, hemoglobin 14.1 gm per cent, and hematocrit 42 per cent She was given 62 mg of radio iron 2 days following, hysterotomy was performed Sampling revealed 3.2 per cent of the amount fed in circulation

Case 25 V G (Hospital No 164558) Age 41 years, para 7 Pregnancy with nephritic toxemia, hypertensive cardio-vascular disease, mild diabetes mellitus Gestation 5 months, therapeutic abortion The patient was relatively asymptomatic There were no signs of decompensation Blood pressure was 188/102 The urine showed 2+ albumin The glomerular filtration was one-third normal She was given 16 mg of radio iron and 2 days later delivered by hysterotomy 3.2 per cent of the dose fed was the amount estimated to be present in the total circulation

Case 26 J T (Hospital No 84390) Age 42 years, para 10, gravida 11 Pregnancy, abnormal with toxemia, pre-eclamptic. Gestation 7 months, therapeutic abortion She complained of ankle edema, headaches, nausea, and vomiting The patient was markedly obese Blood pressure was 230/130 Glomerular filtration 31 per cent Albuminuria was marked She was given 122 mg of radio iron and vomited 1½ hours later At the time of feeding, blood studies showed red blood count 3,600,000, hemoglobin 12.8 gm per cent, hematocrit 37.2 per cent One day later, labor was induced Subsequent blood samples showed 2.5 per cent of the amount fed in the circulation

Case 27 E A (Hospital No 177796) Age 24 years, para 1 Normal pregnancy, contracted pelvis Gestation 8.5 months, cesarean section Blood studies red blood count 3,250,000, hemoglobin 12.5 gm per cent, hematocrit 38 per cent 3 hours before delivery she was given 1.9 mg of radio iron, 16.4 per cent of the amount fed was estimated to be in the circulation

Case 28 L K (Hospital No 169989) Age 21 years, primipara, full term, normal pregnancy At the time of feeding, red blood count was 3,070,000, hemoglobin 9.8 gm per cent, hematocrit 24.8 per cent 63 mg of radio iron was given and spontaneous delivery occurred 10 days later The blood samples revealed 3.9 per cent of the amount fed in the circulation

Case 29 E S (Hospital No 173986) Age 30 years, primipara, full term Normal pregnancy Blood studies red blood count 4,100,000, hemoglobin 13.1 gm per cent, hematocrit 31.7 per cent 33 hours following feeding of 0.9 mg of radio iron, the patient was delivered spontaneously 3.3 per cent of this dose was the estimated amount found in the circulation

Case 30 E M (Hospital No 148022) Age 32 years, para 2, term Normal pregnancy Blood studies red blood count 4 000,000, hemoglobin 11.5 gm per cent hematocrit 37.5 per cent. She was given 5 mg of radio iron, with delivery occurring spontaneously 15 minutes later. The maternal blood on subsequent sampling showed 2.9 per cent of the dose in circulation.

Case 31 E W (Hospital No 145651) Age 23 years para 2, full term. Funnel pelvis, cesarean section Blood studies red blood count 4,100,000 hemoglobin 10.6 gm per cent, hematocrit 34.2 per cent. She was given 93 mg of radio iron and 20 hours later was delivered by cesarean section. 2.2 per cent of the amount fed was estimated to be in the circulating red cells.

TABLE 2
Radioactive Iron in Pregnancy

Case	Gestation	Weight	Esti- mated red cell mass	Hema- tocrit at feeding	Hema- tocrit at sam- pling	Interval between feeding and sam- pling	Dose iron	Per cent amount fed per 100 ml. red cells	Per cent amount fed circulat- ing esti- mated
	<i>mos</i>	<i>kg</i>	<i>ml</i>	<i>per cent</i>	<i>per cent</i>	<i>days</i>	<i>mg</i>	<i>per cent</i>	<i>per cent</i>
21 M C.	2	59.4	1490	43.3	41.7	150	16	0.18	2.7
22 P F	3	88.6	1820	37.5	34.3	10	53		4.9
23 J F	4	77.6	2070	43.7	44.3	200	122	0.20	4.2
24 E. L	4.5	64.8	1440	42.0	37.0	17	62	0.26	3.2
25 V G	5	90.6	2100	38.6	38.5	150	16	0.15	3.2
26 J T*	7	100.4	2480	37.2	41.1	200	122	0.37±	2-5
27 E. A	8.5	63.0	1490	38.0	39.5	45	1.9	1.1	16.4
28 L. K	Term	74.8	1510	24.8	33.6	22	63	0.25	3.9
29 L. S		73.6	1710	31.7	38.7	44	0.9	0.19	3.3
30 E M	"	62.0	1420	37.5	38.2	6	5.0	0.20	2.9
31 E. W		68.2	1655	34.2	40.6	10	93	0.13	2.2
32 J B		57.6	1200	40.3	34.8	43	20	1.87	22.4
33 A M		84.4	2380	43.4	46.9	7	20	0.07	1.6
34 A C.		65±	1125±	32.1	28.8	13	14	2.46	27.7

* Vomited 1.5 hours after feeding

Case 32 J B (Hospital No 178277) Age 35 years, primipara, full term. Normal pregnancy Blood studies red blood count 4 150 000, hemoglobin 12.4 gm per cent, hematocrit 40.3 per cent. She was given 20 mg of radio iron and was delivered spontaneously 40 minutes later. Of this dose 22.4 per cent was estimated to be in the circulation.

Case 33 A. M (Hospital No 166168) Age 23 years para 2 full term. Normal pregnancy Breech delivery Blood studies red blood count 4,670,000, hemoglobin 13.5 per cent hematocrit 43.4 per cent. 20 mg of radio iron was given 20 minutes later she was delivered spontaneously. 1.6 per cent of the dose fed was estimated to be in the circulation.

Case 34 A C (Hospital No 180768) Age 27 years, primipara full term. Normal pregnancy with small pelvis cesarean section 110 minutes after feeding 14

nephritic toxemia with chronic nephritis The first pregnancy was complicated by pre-eclampsia During the present pregnancy, she developed headaches, nausea, vomiting, ankle edema, and albuminuria Blood pressure was 125/90 Retrograde x-ray studies showed bilateral hydronephrosis At the time of feeding, the red count was 4,430,000, hemoglobin 14 gm per cent, hematocrit 43.7 per cent She was given 122 mg of radio iron and the pregnancy was interrupted 2 days later Sampling revealed 4.2 per cent of the amount fed appearing in the circulation

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Case 31 E W (Hospital No 145651) Age 23 years, para 2 full term Funnel pelvis cesarean section. Blood studies red blood count 4,100 000, hemoglobin 10.6 gm per cent, hematocrit 34.2 per cent. She was given 93 mg of radio iron and 20 hours later was delivered by cesarean section 2.2 per cent of the amount fed was estimated to be in the circulating red cells.

TABLE 2
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	mo	kg	ml.	per cent	per cent	days	mg	per cent	per cent
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Case 34. A C (Hospital No 180768) Age 27 years primipara, full term. Normal pregnancy with small pelvis, cesarean section 110 minutes after feeding 14

mg of radio iron, cesarean section was done. Of this dose, 27.7 per cent was estimated to be in circulation.

Table 2 and the related brief clinical histories indicate that as a rule the pregnant woman absorbs more radio iron than normal controls. Two exceptions are noted with normal iron absorption (cases 29 and 33) where we assume that iron stores were abundant in spite of the demands of the fetus. Three cases show maximal or high absorption (cases 27, 32, and 34) but the red cell hematocrits do not correspond as a moderate grade of anemia is present in case 34 but not in cases 27 and 32. We may say that most of these pregnant women show from 2 to 10 times the absorption of radio iron one might expect in a normal individual.

DISCUSSION

To evaluate the results summarized in Tables 1 and 2 it is necessary that several points be kept in mind. The unavoidable variation in dosage would be expected to result in different percentage utilization even in the same individual under the same conditions. This was pointed out by Whipple and Robschey-Robbins (12) in studying the effect of feeding iron over periods of 2 weeks on the hemoglobin production of anemic dogs. They found that increasing the iron dosage 10 times (i.e. from 40 mg per day to 400 mg per day) resulted in the utilization of about twice as much of the metal. This meant a drop in percentage utilization from 30 to 35 per cent to 5 to 7 per cent. Employing the radioactive isotope of iron, Hahn, Ross, Bale, and Whipple (8) showed that the per cent utilization ranged from 60 per cent with a single dose of 1.2 mg of radio iron to 3.2 per cent with a dose of 115 mg when iron depleted anemic dogs were studied. Since the time of the last mentioned report considerably more data have accumulated and it is possible to employ this experience in the proper evaluation of the effect of single doses even though there is appreciable variation in the amount of iron used.

It is reasonably certain that the healthy adult woman must absorb more iron than the healthy male, as we cannot assume that she excretes less iron through the intestinal tract than the male and she must replace the menstrual hemoglobin loss which averages 6 to 9 gm hemoglobin or 20 to 30 mg Fe per month. Whether this excess iron intake could be demonstrated by careful study of groups of healthy males and females by use of radio iron remains for the future.

The *pregnant woman* at least in the late months of pregnancy does show *increased intake of radio iron*, although there are great variations and a few cases of normal iron intake (Table 2). This intake of iron bears no relation to the red cell hematocrit as we observe maximal absorption in cases with low, and again, with high hematocrit (cases 32 and 34, Table 2). We note a normal

absorption with a low red cell hematocrit (case 29) and with a high red cell hematocrit (case 33)

Such evidence as we have in this laboratory, published and unpublished, indicates that the *reserve stores of iron are usually low* in the liver and presumably in other body stores (spleen and marrow) late in pregnancy. It is reasonable to suppose that the insistent demands of the growing fetus deplete the maternal stores even without any significant anemia. Such cases should absorb radio iron readily and almost certainly do so. On a different dietary régime the pregnant woman might keep her reserve stores at an adequate level and absorb only the normal amount of radio iron, that is approximately 1 per cent or less of the iron fed.

The evidence from this group of pregnancy cases is in harmony with other clinical evidence (Table 1) and experimental evidence in the dog—that the reserve stores of iron are of more importance than the hemoglobin level in determining the amount of iron absorption. The intestinal mucosa has the power of discrimination and can take iron or leave it, but the mechanism of this peculiar capacity is obscure.

SUMMARY

Radio iron is a tool which makes iron absorption studies quite accurate in dogs and reasonably satisfactory in human beings. This method is vastly superior to others previously used.

Normal human pregnancy without significant anemia may show active radio iron absorption—16 to 27 per cent of iron intake. The pregnant woman as a rule shows 2 to 10 times the normal absorption of radio iron.

Diseased states in which *iron stores* are known to be *very abundant*—pernicious anemia, hemochromatosis, familial icterus, and Mediterranean anemia—show very little absorption, probably less than normal. This is in spite of a severe anemia in all conditions except hemochromatosis.

Chronic infections in spite of anemia show no utilization of radio iron, whether it may be absorbed or not.

Leukemia shows little utilization of radio iron in red cells in spite of absorption (autopsy), probably because of white cells choking the red marrow.

Polycythemia shows very low values for iron absorption as do normal persons. Two pregnant women showed only normal iron absorption.

We believe that *reserve stores of iron* in the body, rather than anemia, control iron absorption. This control is exerted upon the gastro-intestinal mucosa which can refuse or accept iron under various conditions.

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STUDIES IN RODENT POLIOMYELITIS

III. EXPERIMENTAL POLIOMYELITIS IN GUINEA PIGS PRODUCED WITH THE MURINE STRAIN OF SK POLIOMYELITIS VIRUS*

By CLAUS W. JUNGEBLUT, M.D., ROSE R. FEINER, Ph.D., AND
MURRAY SANDERS, M.D.

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York)

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In earlier attempts to transmit the murine strain of SK poliomyelitis virus from mice to guinea pigs active virus could be recovered from intracerebrally injected guinea pigs up to 96 hours after injection (1). Meanwhile other observations served to indicate that some virus multiplication occurred in tissue cultures prepared with embryonic guinea pig brain. For the above reasons we ventured to predict "the possibility of training the infectious agent to produce ultimately disease in that rodent as well." It is therefore of interest to record that renewed efforts, beginning with the 70th mouse passage, to transmit the murine virus from paralyzed mice to guinea pigs resulted in the occurrence of frank paralysis in the latter animal (2). The present communication sets forth in detail the various experimental aspects of this guinea pig paralysis.

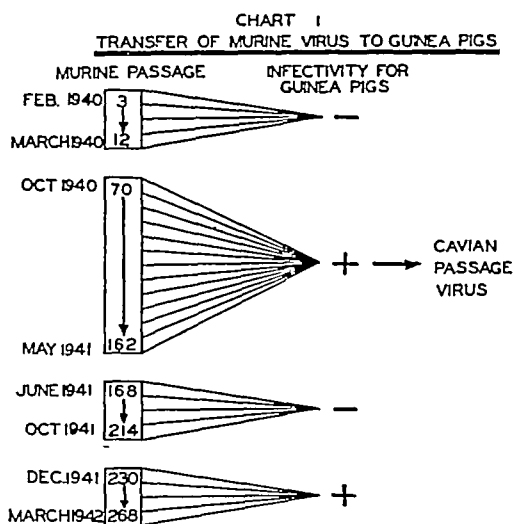
The data presented deal with the adaptation of the murine virus from mice to guinea pigs and describe the symptomatology of the disease thus produced. There are also included certain facts concerning the properties of the guinea pig virus and its manifestations in this host. In addition, the immunity mechanism, which develops in the guinea pig as the result of this infection, was studied. Finally, the behavior of the guinea pig virus in *rhesus* monkeys was made the subject of careful investigation.

Transfer of Murine Virus from Mice to Guinea Pigs and Establishment of a Fixed Strain of Cavan Virus in Guinea Pigs

The murine virus was maintained by unbroken serial transfer from mouse to mouse during the entire course of this investigation. At various points of this continuous line of mouse passages transmission to guinea pigs was attempted by injecting the latter animals with a standard dose of 0.1 cc. of 10 per cent brain suspensions prepared from paralyzed mice. As stated before, virus obtained from several early mouse passages (3rd to 12th mouse passage) proved ineffective in bringing about paralysis in guinea pigs. However, numerous transfers

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incubation period has been as long as from 2 to 3 weeks, once the virus is fixed in the guinea pig, as the result of prolonged passage, the disease develops with marked regularity between the 3rd and 6th day. No symptoms are noted, as a rule, during the incubation period. Observations extending over more than 175 infected guinea pigs indicate that some animals may show a preparalytic fever of 104 to 105°F which drops with the onset of paralysis. This febrile reaction, when present, seems well enough defined, but individual variations are such as to preclude any pathognomonic fever curve. Paralysis sets in abruptly, often overnight, and is complete within a few hours, characteristically involving the hind legs more often than the front legs. This condition in a majority of animals progresses to prostration with a terminal fatal issue. Occasionally cavian virus may produce a syndrome wherein infected animals, after a some-



what longer incubation period, show salivation and become marasmic, but exhibit no distinct flaccid paralysis. In two instances animals showing such a syndrome were sacrificed and blood as well as suspensions of spleen, brain, and cord were inoculated intracerebrally or intraperitoneally into mice and guinea pigs. Blood and spleen produced no symptoms whatsoever, whereas all mice and guinea pigs injected with brain or cord developed typical flaccid paralysis in from 3 to 4 days. The conclusion seems justified, therefore, that the described aberrant syndrome was not caused by the activity of any possible viral contaminant, but that the encephalitic symptoms should be ascribed to an unusual persistence and localization of the cavian virus in the brain of infected guinea pigs. This conclusion is further supported by careful pathological examination of the central nervous system of such guinea pigs which disclosed the presence of a polioencephalitis, together with characteristic lesions in the

anterior horn of the spinal cord. A detailed report of the pathology of the guinea pig paralysis will be found in another paper of this series of communications (4).

Routes of Infection in Guinea Pigs

During the two periods that murine virus could be successfully transmitted from mouse to guinea pig the incidence of paralysis in the latter animal was

TABLE II

Incidence of Paralysis in Guinea Pigs Following Infection with Murine Virus (70th to 168th Mouse Passage) or Cavian Virus (3rd and 21st Guinea Pig Passage) by Different Routes

Route of Infection	Virus	Dose	No of guinea pigs	Result	
				Paralysis	No paralysis
Intracerebral	Murine*	0.1	36	32	4
	Cavian†	0.1	7	7	0
Intravenous	Murine	1.0	3	2	1
	Cavian	1.0	3	2	1
Intraperitoneal	Murine	2.0	6	4	2
	Cavian	1.0-2.0	6	0	6
Subcutaneous	Murine	2.0	3	1	2
	Cavian	1.0-2.0	6	0	6
Intranasal	Murine	1.0	5	0	5
	Cavian	0.2-1.0	6	0	6
Oral	Murine	2.0‡	58	0	58
	Cavian	1.0-2.0‡	5	0	5

* 10 per cent mouse brain suspension.

† 10 per cent guinea pig brain and cord suspension.

‡ On 3 consecutive or alternate days.

uniformly high, with an average infectivity of 90 and 75 per cent, respectively. Paralysis was also obtained by intravenous, intraperitoneal, or subcutaneous injection, although the last route proved definitely the least effective. In intranasal instillation or oral administration of murine virus have consistently failed to evoke any unmistakable signs of paralysis (Table II).

When virus is employed which has been passed through guinea pigs for several generations paralysis can readily be produced by intracerebral injection but not by intraperitoneal, subcutaneous, intranasal, or oral administration. However,avian fixed virus of the 21st generation was capable of inducing

paralysis upon intravenous as well as intracerebral inoculation, the same material when injected into guinea pigs by the intraperitoneal, subcutaneous, nasal, or oral route again failed to cause any paralytic symptoms (Table II)

The inability of murine virus to infect guinea pigs by oral administration stands in marked contrast to the results obtained in mice. This problem was therefore made the subject of further study. It was first thought that the ingestion of certain other substances along with the virus might render the latter infectious from the gastrointestinal tract. Thus, in one experiment, feeding of murine virus was followed by feeding of egg yolk, gastric mucin, *coli* bacteriophage, or of a suspension of triturated mouse intestine. Attempts were also made to bring about changes in the condition of the animal which might facilitate invasion of the infectious agent. One series of guinea pigs, prior to oral administration of murine virus, was therefore injected with sublethal doses of diphtheria toxin, while another series had previously been maintained on a scorbutic diet. Suffice it to say that none of these methods were of any avail in rendering the murine virus infectious for guinea pigs by the gastrointestinal route.

Titration of Virus

Titration of the infectivity of murine virus (141st to 144th mouse passage) in guinea pigs showed that dilutions of mouse brain suspension up to 1:500 were effective in producing paralysis following intracerebral injection. On the other hand,avian virus of the 13th guinea pig passage seemed to possess much less potency since brain and cord suspensions, injected intracerebrally, failed to produce paralysis in dilutions above 1:10. A more recent titration of avian virus in its 38th guinea pig passage, however, indicates a substantial increase in virulence in that brain and cord dilutions up to 1:500 proved infective.

Titration of the infectivity of avian virus (brain and cord suspensions of the 25th and 31st guinea pig passages) for mice revealed that guinea pig passage apparently reduces the mouse virulence of the virus, titration end points in mice being 10^{-8} , as determined by intracerebral test. When transferred further to new mice, however, the virus proved again infective to a titer of 10^{-7} . Inasmuch as this titer almost reached the virulence level of mouse passage virus (10^{-8} by the intracerebral route), passage through mice seems to cause a rapid return of the avian virus to its original murine character.

Distribution of Virus in Guinea Pigs

Pathological observations (2, 4) had left little question that the lesions in paralyzed guinea pigs are strictly confined to the central nervous system, particularly the anterior horn cell in the spinal cord. The marked neurotropism of the virus in this animal is further substantiated by the results of experiments in which the distribution of murine or avian passage virus was studied in intra-

cerebrally infected guinea pigs. For this purpose nervous and non nervous tissues of infected guinea pigs, at the height of paralysis, were transferred to mice. Mice were used in these tests because they were known to react to small doses of virus with great regularity. It was found that the transfer to mice of brain and cord from paralyzed guinea pigs always resulted in typical paralysis after a short incubation period (2 to 4 days), indicating the presence of large amounts of virus in such tissues. By contrast, transfer of blood, spleen, or liver was negative in nearly all instances (Table III). The technique employed throughout these experiments consisted in the intracerebral or intraperitoneal

TABLE III

Distribution of Virus in Guinea Pigs Following Administration of Murine or Cavian Virus

Route of infection	Tissues examined for virus content									
	Brain and cord		Spleen		Liver		Blood		Feces	
	No. of animals examined	Presence of virus	No. of animals examined	Presence of virus	No. of animals examined	Presence of virus	No. of animals examined	Presence of virus	No. of animals examined	Presence of virus
Intracerebral*										
Murine	7	+	2	±	1	—	1	—	5†	—
Cavian	21	+	8	—	6	—	6	—		
Oral‡										
Murine	28	—					10	—	45§	±
Cavian	1	—							6‡	—

+ = virus present in all instances ± = virus present irregularly — = virus not present.

* Guinea pigs examined were sacrificed during incubation period or at height of paralysis.

† One pooled sample tested.

‡ Guinea pigs examined were sacrificed at intervals of 1 to 14 days after oral administration of virus.

§ Nine pooled samples tested. Three samples were positive, 6 were negative

injection of mice with 0.03 cc. or 0.1 cc. of 10 per cent suspensions of the various tissues, blood was either injected intraperitoneally (0.5 to 1 cc.), freshly drawn, or intracerebrally (0.03 cc.) and intraperitoneally after laking or heparinization. These findings stand in sharp contrast to analogous observations in mice which had revealed a systemic distribution of the virus in that animal, at least during certain phases of the infectious process.

The possibility of a centrifugal spread of the virus from the infected central nervous system was next examined by testing the feces of paralyzed guinea pigs for virus content. In these experiments, 10 per cent saline suspensions of feces were filtered through a N Berkefeld candle and then injected intraperitoneally into mice in amounts of 0.2 to 0.5 cc. In several tests it proved impossible to

recover any virus from the feces of guinea pigs paralyzed by intracerebral injection of either murine or cavian virus. However, active virus could be recovered, on three occasions, from the feces of guinea pigs following oral administration of murine virus. None of these animals had shown any paralytic symptoms and transfer of brain and cord to mice indicated that their central nervous system was demonstrably free from virus. Virus has not been encountered, in repeated tests, in the feces of normal guinea pigs.

Cultivation of Cavian Virus in Vitro

Repeated attempts to grow cavian virus in tissue culture were unsuccessful until virus of the 15th guinea pig passage was inoculated into embryonic mouse brain tissue culture. This type of tissue culture and the technique of subculturing are fully described in another paper of this series of communications (5). When tested intracerebrally in guinea pigs, undiluted tissue culture fluid of the 12th *in vitro* subculture paralyzed 1 of 2 animals, whereas a similar inoculation of the 30th subculture gave negative results. Both subcultures, however, proved infectious for mice, the latter to a titer of 10^{-5} by the intracerebral route. These preliminary experiments suggest that cavian virus can be grown in tissue culture but that optimum conditions for its propagation, with full maintenance of guinea pig virulence, have as yet not been obtained. Possibly embryonic guinea pig brain rather than mouse brain may make a better substrate for *in vitro* cultivation of this virus.

Serological Tests with the Cavian Virus

Neutralization tests were carried out in order to study the nature of the cavian virus with the help of serological methods. The object of this work was, first, to establish, if possible, the identity between murine and cavian virus, and, secondly, to determine whether cavian virus was antigenically related to authentic poliomyelitis virus. These tests therefore consisted of (1) experiments in which immune rabbit sera prepared against cavian or murine passage virus were examined for their ability to inactivate the homologous and heterologous viruses in mice and guinea pigs, (2) experiments in which guinea pig convalescent serum, monkey SK and Aycock convalescent sera, and anti-poliomyelitis horse sera¹ were tested for neutralizing power against cavian virus in guinea pigs. Similar tests were carried out, for control purpose, with a hyperimmune rabbit serum against the virus of lymphocytic choriomeningitis² and with normal sera from rabbits, guinea pigs, monkeys, and horses.

It was clearly shown that anticavian rabbit immune serum possesses strong

¹ These sera were obtained through the courtesy of Dr J. A. Toomey from the City Hospital, Cleveland.

² This serum was obtained through the courtesy of Dr J. E. Smadel from Dr Rivers' laboratory.

virucidal power against murine virus in mice since the serum neutralized the maximum dose of virus used, i.e. 10^{-1} . These tests were run by intraperitoneal injection of mice with virus-serum mixtures, as described in another paper (3). The reverse is also true, namely that cavian virus in guinea pigs is completely

TABLE IV
Neutralization in Vitro of Cavian Virus in Guinea Pigs by Various Antisera

Serum	Result
Anticavian rabbit serum	0/3
Antimurine rabbit serum 1st test	0/3
2nd test	0/3
3rd test	1/4
Antilymphocytic choriomeningitis rabbit serum 1st test	2/3
2nd test	3/4
Normal rabbit serum 1st test	3/3
2nd test	3/4
Guinea pig convalescent serum	0/3
Normal guinea pig serum 1st test	2/3
2nd test	3/4
SK monkey convalescent serum 1st test	0/3
2nd test	3/4
Aycock monkey convalescent serum 1st test	2/3
2nd test	0/3
3rd test	4/4
Normal monkey serum 1st test	3/3
2nd test	4/4
Antipoliomyelitis horse serum	
Untreated (096464-G)	7/7
Concentrated fraction (097607 A)	0/7
Normal horse serum	6/6

Numerator = number of guinea pigs with symptoms or paralysis. Denominator = number of guinea pigs injected.

Technique—0.5 cc. of 20 per cent guinea pig brain and cord suspension was mixed with 0.5 cc. of undiluted serum after incubation for 1 hour at 37°C. 0.1 cc. of each mixture was injected intracerebrally into several guinea pigs.

inactivated by antimurine rabbit immune serum (Table IV). This neutralization is as marked as that occurring with homologous anticavian rabbit immune serum or with serum obtained from convalescent paralyzed guinea pigs. Thus, the serological identity of murine and cavian virus can be said to be firmly established. The results with the various monkey convalescent sera are irregular and difficult of interpretation, especially in view of the fact that parallel neu

tralization tests with the same sera against their corresponding strains of monkey poliomyelitis virus had not been carried out. However, one of two potent anti poliomyelitis horse sera (concentrated fraction) gave complete neutralization in repeated tests, the same serum had previously neutralized murine virus in mice (3). In contrast herewith, neutralization of cavian virus was not obtained with anti lymphocytic-choriomeningitis rabbit immune serum nor with any of the several normal sera.

Immunity Phenomena of the Disease in Guinea Pigs

During the course of this investigation there were available for further study a number of guinea pigs which had survived infection by various routes with either murine or cavian passage virus without showing any paralytic symptoms. These guinea pigs were in no way convalescent animals but had remained completely free from any objective signs of disease. Three to 6 weeks after their first injection these symptomless guinea pigs were reinfected intracerebrally either with potent murine or cavian passage virus. The results are listed in Table V.

Upon considering the data as a whole it will be gathered that of a total of 70 animals which had failed to show symptoms following a first inoculation with either murine or cavian virus, 46 were solidly immune to reinfection with the same viruses whereas 24 proved susceptible, all 33 accompanying control animals succumbed to the disease. The described protection occurred irrespective of whether the guinea pigs had previously received cavian virus or murine virus. It will be noted, however, that guinea pigs which had received the initial virus injection intracerebrally were better protected than those which had previously been injected by the nasal or oral route³.

The above observations indicate that murine or cavian virus, when causing an inapparent infection in guinea pigs, is capable of leaving the animal in a state of immunity which is of considerable proportions. The possibility must be considered that this resistance may be, in some way, connected with persistence of the virus. For active virus, as determined by transfer to mice, could be recovered, on one occasion, from symptomless guinea pigs injected intracerebrally with cavian passage virus as late as 3 weeks following such injection.

It became of interest to investigate further the mechanism of this "latent immunity" by determining to what extent the tissues or the serum of resistant guinea pigs possessed the power to inactivate virus. The first experiment included a group of 8 guinea pigs, none of which had shown any symptoms fol-

³ It was found that the injection of massive doses of Theiler's virus in guinea pigs fails to produce any clinical signs of disease, even though the virus may be recovered as late as 72 hours after intracerebral injection from the central nervous system of such animals. It is of interest to note in this connection that such symptomlessly infected guinea pigs proved fully susceptible to reinfection with cavian virus.

TABLE V

Reinfection with Murine or Cavian Virus of Guinea Pigs Giving a Previous History of 'Symptomless Infection'

Experiments	Previous history of guinea pigs	No. of guinea pigs	Virus used for reinfection	Results	
				Paral-ysis	No paral-ysis
Experiment 1 1/7/41	Murine virus I.C.	1	Murine virus I.C.	0	1
	Cavian "	9	"	3	6
	Normal controls	6	"	6	0
Experiment 2 1/24/41	Murine virus I.C.	1	"	0	1
	Cavian	5	"	3	2
	Normal controls	5	"	5	0
Experiment 3 3/7/41	Murine virus I.P.	1	" "	0	1
	" S.C.	1	" "	0	1
	" orally	6	" "	3	3
	Normal controls	2	"	2	0
Experiment 4 4/4/41	Murine virus orally	6	"	2	4
	Cavian I.C.	2	"	0	2
	Normal controls	2	"	2	0
Experiment 5 5/17/41	Cavian virus I.C.	3	"	1	2
	Normal controls	2	"	2	0
Experiment 6 9/25/41	Murine virus I.C.	4*	Cavian virus I.C.	1	3
	Cavian	7		1	6
	" I.P.	2		1	1
	" S.C.	3		1	2
	" I.N.	3		3	0
	" orally	2		2	0
	Normal controls	5		5	0
Experiment 7 10/8/41	Murine virus I.C.	5*		0	5
	Normal controls	4		4	0
Experiment 8 11/21/41	Murine virus I.C.	3*	"	0	3
	Normal controls	4		4	0
Experiment 9 2/10/42	Murine virus I.C.	3	"	0	3
	Cavian orally	3		3	0
	Normal controls	3		3	0

I.C. = intracerebrally S.C. = subcutaneously

I.P. = intraperitoneally I.N. = intranasally

* These guinea pigs had received murine virus during the time that negative transfers were obtained from mouse to guinea pig

lowing injection with murine virus, and another group of 7 guinea pigs which were convalescing from an attack of murine paralysis, a third group of 4 normal guinea pigs was added for control purpose. All 19 guinea pigs received an intracerebral dose of 0.1 cc. of a 10 per cent suspension of potent murine virus. At intervals varying from 24 to 72 hours these animals were sacrificed and their brain and cord transferred to mice. No virus, or at best only traces of virus, were present in the brain or cord of either symptomless or convalescent animals, whereas active virus could regularly be recovered at 48 to 72 hour intervals from the nervous tissue of infected normal guinea pigs. The results of these tests suggest, first, that the central nervous system of symptomless guinea pigs is capable of disposing of virus in a highly effective manner and, second, that this disposal is analogous to the mechanism which operates in convalescent animals. The protection, therefore, appears to be essentially the same, irrespective of whether the guinea pig had or had not had paralysis before.

In a second experiment the sera of latently immune guinea pigs were examined for their power to inactivate murine virus *in vitro*. These sera were obtained from various groups of symptomless guinea pigs, *i. e.* guinea pigs which had been fed murine virus, or guinea pigs which had received either murine or cavian virus intracerebrally without developing paralysis. A total of 11 sera, pooled in three lots according to origin, was thus examined, normal guinea pig sera were included for control purposes. The results of these neutralization tests showed that the pooled sera of latently immune guinea pigs, without exception, were strongly virucidal for murine virus as determined by intraperitoneal tests in mice, inasmuch as they neutralized doses of virus as high as 10^{-2} . By contrast, no virucidal power could be detected in normal control sera which failed to neutralize virus dilutions up to 10^{-5} .

When the two experiments are taken together there can be little doubt that guinea pigs rendered latently immune to virus by symptomless infection are endowed with a well developed protective mechanism, which can be demonstrated, on the one hand, by the power of nerve tissue to dispose of virus *in vivo*, and, on the other, by the ability of serum to inactivate the infectious agent *in vitro*. While no unequivocal distinction can be made between cellular or humoral factors involved, the nature of the clearing mechanism invites further study and clarification. What seems to be of particular interest, however, even at this early stage of the problem, is the fact that neutralizing antibodies were readily found in the sera of guinea pigs following oral administration of the virus, even though no active virus could be discovered in the central nervous system of such animals.

Non-Specific Age Resistance of Guinea Pigs to Infection with Murine Virus

The next experiments deal with the question whether non-specific protection against cavian virus can be demonstrated in guinea pigs as the result of increased resistance with age. As is well known, many neurotropic viruses,

when injected by peripheral routes into adult animals, encounter physiological barriers which impede their further travel to the central nervous system. As far as poliomyelitis is concerned, this question has not yet been put to a satisfactory experimental trial because small laboratory animals susceptible to this virus have heretofore not been available. The opportunity was therefore seized to investigate this problem in guinea pigs.

A group of 33 guinea pigs, weighing 600 gm. or more, received murine virus by various routes, 8 intracerebrally, 10 intravenously, 10 interperitoneally, and 5 subcutaneously. The incidence of paralysis among these animals, following intravenous or intraperitoneal injection, did not differ materially from that observed in young guinea pigs. There was a suggestion, however, that the older animals were slightly more resistant to subcutaneous and, perhaps, even to intracerebral inoculation since by the former route only 3 of 8 came down with paralysis and by the latter none of 5 animals. Admittedly, the number of guinea pigs used was too small and the experiment not sufficiently complete to yield conclusive evidence. However, it is interesting in this connection to mention that neutralizing substances for this virus are not present in the sera of normal adult guinea pigs (5 sera tested) in contrast with the sera of symptomless infected animals which regularly contain such antibodies. The problem of age resistance in the guinea pig obviously requires further study, with consideration of the pathogenesis of the disease following different routes of injection, and the use of graded infecting doses, such as was done for murine virus in mice (3).

Pathogenicity of Cavian Virus for Rhesus Monkeys

The monkey pathogenicity of the guinea pig virus was studied by inoculating a number of *rhesus* monkeys with the brain or cord of guinea pigs paralyzed by passage virus, the usual dose was 1 cc. of 10 per cent suspensions injected intracerebrally. Such transfers from guinea pig to monkey were carried out with every consecutive passage during the first two lines of virus propagation in guinea pigs, and with the 8th and 21st passage of the third line. The results are given in Table VI.

It will be seen that of a total of 35 monkeys which had received guinea pig passage virus intracerebrally, 26 failed to respond with any manifest symptoms of disease, save for a transient fever and occasional awkwardness in movements. Intracerebral reinfection with SK poliomyelitis virus which paralyzed all of 12 accompanying normal controls failed to produce paralysis in 5 of 19 such symptomless monkeys. There remain 9 monkeys in which the injection ofavian virus was followed by various signs of definite involvement of the central nervous system. Thus, 5 animals developed a characteristic encephalitic syndrome consisting of coarse tremor, convulsions and facial palsy, 3 showed a paresis of one or more extremities, and 1, on the 8th day after infection, presented a complete flaccid paralysis of the left leg. The clinical picture ob-

served in this latter animal was that of "classical" poliomyelitis, the train of symptoms progressing from a preparalytic fever to paresis and, thence, to frank paralysis, moreover, pathological examination of the central nervous system, upon sacrifice on the 10th day of the disease, revealed typical unilateral poliomyelitic lesions in the lumbar level of the spinal cord

The available data may be summarized by saying that cavian passage virus, in principle, is no more pathogenic for *rhesus* monkeys than is murine passage virus. In other words, a single intracerebral injection of virus usually causes

TABLE VI
Transfer of Guinea Pig Passage Virus to Rhesus Monkeys

Line of propagation in guinea pigs	Guinea pig passage	No. of monkeys injected intracerebrally with guinea pig brain or cord	Result			
			Negative*	Encephalitic syndrome	Paresis	Paralysis
1st	I	2	1		1	
1st	II	7	5	1	1	
1st	III	2	2			
1st	IV	6	3	1	1	1
1st	V	2	1	1		
2nd	I	2	2			
2nd	II	2	2			
2nd	III	2	2			
2nd	IV	8	7	1		
3rd	VIII	1	1			
3rd	XXI	1		1		
1st-3rd	I-XXI	35	26	5	3	1

* Monkeys listed as negative had either no symptoms whatsoever or showed a transient fever (103-107°F) with occasional awkwardness in movements

no manifest symptoms of disease, although certain animals may develop an encephalitic syndrome, with or without localized pareses. In one instance, however, classical poliomyelitis occurred in a monkey following injection with cavian virus obtained from a passage which failed to transfer further to guinea pigs, but was still paralyzing mice. The full significance of these observations for the mechanism of virus adaptation from monkey to rodents, and vice versa, is as yet not immediately apparent.

Recovery of Virus from the Tissues of Poliomyelitis-Convalescent Monkeys Following Injection with Cavian Virus

The results of previous experiments (1) will be recalled which had shown that the tissues of poliomyelitis-convalescent monkeys, when tested 24 to 96 hours

after injection with murine virus, were relatively, if not absolutely, free of the infectious agent. Since a highly effective clearing mechanism had just been demonstrated in the tissues of guinea pigs convalescing from cavian paralysis or latently immune to this virus, it became of interest to investigate the fate of cavian virus after injection into poliomyelitis-convalescent monkeys. A total of 9 monkeys which had survived a previous poliomyelitic infection with SK or Aycock virus were available for this purpose. All showed considerable residual paralysis at the time of this experiment, but the interval between their paralytic attack and the injection of cavian virus varied from 2 weeks to as

TABLE VII

*Recovery of Virus from Tissues of Poliomyelitis-Convalescent Monkeys Following Injection with Cavian Virus As Determined by Transfer to Mice**

Monkey	Interval between paralysis and injection of cavian virus	Route of injection with cavian virus	Recovery of virus from monkey tissues			
			Brain	Cord	Spleen	Blood
AG72 Normal	—	Intracerebral	+	+	+	+
AH18 Convalescent SK	3 wks.		—	—	—	—
AG43 ' "	3½ mos.		+	+	+	+
AG19 Aycock	3½		+	+	+	+
AH43 Normal	—	Intravenous	—	—	+	+
AH54 Convalescent SK	2 wks.		—	—	—	—
AH58 ' Aycock	2 "		—	—	—	—
AG81 ' SK	2 mos.		—	+	+	—
AG83 " "	2		+	+	+	+
AG8 " "	3		—	+	+	+
AG56 ' "	3		+	+	+	+

* All monkeys were sacrificed 72 hours after the injection of cavian virus.

long as 3 months. Cavian passage virus (8 to 12th generation) was injected into these animals, either intracerebrally or intravenously, in doses of 1 cc. or 5 cc., respectively, of a 10 per cent guinea pig brain-cord suspension. Two normal monkeys received the same inoculum. After a uniform interval of 72 hours all animals were sacrificed and brain, cord, spleen, and blood were transferred to mice. The technique was the same as previously employed in similar tests with guinea pigs. The results of this experiment are given in Table VII.

The data given in Table VII show that cavian virus injected intracerebrally into a normal monkey was recovered from the central nervous system as well as from extraneural sites, while the same virus after intravenous injection was recoverable only from spleen and blood. In contrast herewith no virus, irrespective of the route of injection, could be recovered from nervous or non nervous tissue of monkeys shortly after a preceding attack of poliomyelitis,

however, in the later stages of convalescence transfers were positive from practically all tissues tested. It would therefore seem that poliomyelitic paralysis in the monkey is associated with a well developed but transient virucidal mechanism for the elimination of cavian virus.

Immunization of Monkeys with Cavian Virus

A group of 4 monkeys were immunized with cavian passage virus (1st to 5th generation). The virus was administered by subcutaneous injection, in ten equal doses of 7 cc of 10 per cent brain-cord suspensions, over a period of 1 month. At the end of the immunization period these monkeys were tested for immunity by intracerebral injection with 1 cc of a 1:10 dilution of SK monkey virus. Two normal control monkeys received a similar dose of SK virus by the intracerebral route. Both control animals developed partial paralysis. Of the 4 immunized monkeys, 2 developed partial paralysis, 1 showed a transient weakness of the arms and legs, and 1 remained free from any symptoms. The degree of protection in this experiment was certainly not striking.

Interference between Cavian and Monkey Virus in Rhesus Monkeys

One attempt was made to determine whether cavian virus could be used effectively as an interfering agent in blocking poliomyelitic infection in monkeys. Six monkeys were injected intracerebrally with 0.5 cc of RMV virus in a 1:10 dilution. The animals were then divided into two groups of 3 monkeys each, one group receiving intravenously repeated doses of 6 cc of 10 per cent cavian virus (brain-cord suspension of 12th to 19th generation), beginning with the day of infection, the other group receiving the same dose of cavian virus 48 hours after infection. Three control monkeys were injected intracerebrally with 0.5 cc of a 1:10 dilution of RMV virus alone. All animals in this experiment developed prostrating paralysis after an incubation period of 6 to 8 days, except one which had received cavian virus at the 48 hour interval. This monkey survived with slight paralytic involvement of both arms and legs.

The above results furnish no evidence of effective interference between cavian virus and RMV virus in monkeys under the conditions of the test. Possibly the failure was due to the low virulence (1:20) of the cavian virus in its early passages, or to the large dose of RMV virus (1:10) used in this experiment. A final conclusion can only be reached after further tests have been done under more favorable quantitative conditions.

DISCUSSION

In 1910, while studying experimental poliomyelitis in monkeys, Roemer and Joseph (6) called attention to a presumably spontaneous flaccid paralysis which

occurred in approximately 5 per cent of their normal guinea pig stock. The causative agent was proved to be a filterable virus but further identification was not carried out, probably because the virus was lost in its 5th passage through guinea pigs (7). Similar isolated observations, although less documented, were made by Neustaedter (8) in 1913 and by Picard (9) in 1925, both authors interpreting their findings as spontaneous induction of poliomyelitis in guinea pigs following close contact with poliomyelitis-infected monkeys. In the absence of further published records, it must remain undecided, first, whether the described condition actually represented authentic poliomyelitis acquired by chance contact at the implied source of infection, secondly, whether the disease was caused by a known virus, such as lymphocytic choriomeningitis or equine encephalomyelitis, which happened to occur in these guinea pigs with atypical manifestations alone or together with poliomyelitis virus, or, thirdly, whether the authors were dealing with a truly spontaneous infection in guinea pigs that imitates poliomyelitis to the same extent as does Theller's spontaneous encephalomyelitis in mice.

Notwithstanding the above mentioned observations, it has generally been held that the guinea pig is insusceptible to experimental inoculation with human or simian poliomyelitis virus. There seems no reason now to depart from this traditional belief—as far as the power of the virus to induce paralysis is concerned—because attempts to transfer SK poliomyelitis virus directly from monkeys to guinea pigs have uniformly failed to elicit any paralytic symptoms in the hands of Trask and his coworkers (10), as well as in our own experience. However, a new impetus is given to this problem by the data presented in this paper, since adaptation of poliomyelitis virus from monkey to guinea pig apparently succeeded by subjecting the virus to intermediary passage through certain rodents, namely the cotton rat and the white mouse. Confirmatory evidence that the SK strain can be transferred from the cotton rat to the guinea pig has since been presented by Toomey and Takacs (11).

The possibility that the cavian virus described in this paper is a contaminating virus, naturally occurring in guinea pigs, can be safely eliminated because cavian virus is serologically indistinguishable from and in all probability identical with murine virus. It may be assumed, then, that the disease in guinea pigs is the result of a direct transfer of the infectious agent from mouse to guinea pig. Therefore, all other evidence suggesting the poliomyelitic nature of the SK virus in mice (1, 5) is equally applicable to identification of the virus in guinea pigs. Further support comes from the symptomatology and pathology of the guinea pig paralysis, which approaches the picture of poliomyelitis in man and monkey more closely than does the mouse paralysis. To these observations may be added the fact that attempts to transfer cavian virus to *rhesus* monkeys have occasionally resulted in localized pareses and, in one instance, in flaccid paralysis. The lack of protection in monkeys follow-

ing immunization with cavian virus against intracerebral reinfection with SK monkey poliomyelitis virus cannot be weighed too much, since the same procedure would also prove unsatisfactory with authentic strains of monkey poliomyelitis virus. As far as identification by serological methods is concerned, inactivation of cavian virus in guinea pigs by poliomyelitis-convalescent monkey sera is definite enough when it occurs, although the data can hardly be said to be sufficiently convincing to bear much weight. More suggestive evidence comes from neutralization tests with potent antipoliomyelitis horse sera, one of which proved capable of completely inactivating the cavian virus in repeated tests. The trend of these experiments, therefore, is not inconsistent with an assumption of some antigenic relationship between poliomyelitis virus and the cavian strain of virus, although certain differences do undoubtedly exist. It is well to remember in this connection that similar difficulties limit the usefulness of the neutralization test, even when applied to the problem of identifying certain strains of poliomyelitis virus upon fresh isolation from man.

Of collateral interest is the information obtained from attempts to trace the fate of cavian virus in the tissues of normal and poliomyelitis-convalescent monkeys. As far as the available data go they show that paralyzed monkeys, shortly after an attack of poliomyelitis, possess a well marked clearing mechanism for cavian virus which is not present in normal monkeys nor in recovered monkeys during the late stages of convalescence. This ability of poliomyelitis-immune tissues to dispose of cavian virus *in vivo* is analogous not only to the elimination of cavian virus from convalescent guinea pigs, but also to the fate of poliomyelitis virus in monkeys after a paralytic attack of poliomyelitis (12).

Because the study of experimental poliomyelitis for many years has been confined to observations in only one host, *i.e.* the *rhesus* monkey, accumulated knowledge has been necessarily incomplete and, to some extent, misleading. The trend of recent investigations suggests that important differences may exist in the response of other hosts to the same virus, since *cynomolgus* monkeys and chimpanzees were found to be fully susceptible to infection by the gastrointestinal route which is normally ineffective in *rhesus* monkeys. As the opportunities expand for further study of the experimental infection in new hosts, additional channels are opened up that may be expected to afford a clearer insight into the complexity of the human disease. Inasmuch as there is now presumptive evidence that SK poliomyelitis virus can be successfully propagated, as a fixed strain of virus, not only in monkeys but also in white mice and guinea pigs, it becomes of interest to compare the similarities and dissimilarities in the response of the three hosts to the same infectious agent. In the first place, one is impressed by the decided neurotropism of the virus in *rhesus* monkeys and in guinea pigs which stands in marked contrast to its wide distribution in mice. Further differences exist in the effectiveness of various avenues of infection. Thus, *rhesus* monkeys are constantly susceptible only

to intracerebral injections of the virus, guinea pigs may acquire the disease by intracerebral and intravenous injection, while mice succumb to intracerebral infection as well as to introduction of the virus by all peripheral routes, including feeding and nasal instillation. In keeping with these observations the virulence of the virus varies considerably among the three hosts, whereas titers between 1:100 and 1:500 represent the approximate end points of activity in *rhesus* monkeys and in guinea pigs, respectively, the virus reaches levels of potency in mice that lie in the neighborhood of 1:1 billion. The higher potency in mice, together with an apparently broader basis of cellular attack, may be the reason why *in vitro* cultivation has so far clearly succeeded only with murine virus.

One phenomenon, outstanding in the course of this investigation, which deserves special consideration is the apparent cyclic variation in the transmissibility of the infectious agent from mice to guinea pigs. It will be recalled that while murine virus was being serially propagated through unbroken mouse passages andavian virus through similar unbroken guinea pig passages, phases of positive transfer from mouse to guinea pig alternated with phases of negative transfer. It is impossible to say at present whether these fluctuations in pathogenicity were caused by periodic changes inherent in the biological nature of the virus, or whether they reflect alterations of a possibly seasonal character in susceptibility of the guinea pig which are brought out only when the virus is forced to adapt itself from one host to another. That the non-paralytic transfers cannot be regarded as "missed infections" is amply shown by the protection against reinfection which results from such symptomless attacks. A similar protection is also demonstrable in guinea pigs which have remained symptomless following injection of murine or avian passage virus by ineffective routes. It would therefore seem as if the virus in guinea pigs may either assume the form of a manifestly paralyzing agent or of an agent which causes latent infection with subsequent development of a strong cellular and humoral immunity. The analogy between such experimentally controlled observations and the epidemiology of poliomyelitis in man is striking and one will look to further elucidation of this problem as a promising lead for a better understanding of the pathogenesis of the human disease.

CONCLUSIONS

1. Murine SK poliomyelitis virus has been transferred from mouse to guinea pig with the establishment of a fixed strain of avian passage virus.

2. The disease thus produced in guinea pigs is characterized by the occurrence of flaccid paralysis. Typical poliomyelitic lesions are found in the anterior horn of the spinal cord.

3. Guinea pigs are susceptible to infection with murine virus by the intracerebral, intravenous, intraperitoneal, and subcutaneous route, avian passage

virus produces paralysis only upon intracerebral or intravenous injection. Neither virus paralyzes guinea pigs by feeding or nasal instillation.

4 The potency of the virus (murine or cavian) in guinea pigs is considerably lower than in mice and compares with the titer of the original SK strain in monkeys. In paralyzed guinea pigs the virus is found only in the central nervous system and not in extraneural sites, such as blood or abdominal viscera.

5 Attempts to cultivate cavian passage virus in tissue culture have yielded evidence of some *in vitro* propagation but no passage virus has as yet been obtained by this method.

6 Cross neutralization tests with cavian passage virus in guinea pigs and with murine virus in mice have established the serological identity of the two viruses. Inactivation of cavian passage virus in guinea pigs by poliomyelitis-convalescent monkey sera is irregular. Complete neutralization has been obtained with a concentrated poliomyelitis horse serum.

7 Resistance to reinfection with potent virus can be demonstrated in convalescent guinea pigs as well as in guinea pigs which have survived a symptomless infection with either murine or cavian virus. This immunity is demonstrable by the power of the serum of such animals to neutralize the virus *in vitro* and by the ability of nerve tissue to dispose *in vivo* of the infectious agent.

8 Cavian passage virus has a limited pathogenicity for *rhesus* monkeys. Of a total of 35 monkeys injected intracerebrally with guinea pig passage virus, 26 failed to respond with any manifest symptoms of disease, 8 monkeys showed various signs of definite involvement of the central nervous system consisting of tremor, convulsions, facial palsy, and localized pareses, 1 monkey developed typical flaccid paralysis.

9 Following injection with cavian virus the virus may be recovered from the tissues of normal monkeys but not from the tissues of convalescent monkeys shortly after a paralyzing attack of poliomyelitis due to SK or Ayrcock virus.

10 Immunization of monkeys with early cavian passage virus by the subcutaneous route has given no clear-cut evidence of protection against intracerebral reinfection with SK poliomyelitis virus. Neither has there been any evidence of effective interference in monkeys injected intravenously with early cavian passage virus and intracerebrally with RMV poliomyelitis virus.

11 The bearing of the experimental data upon the epidemiology of the human disease is discussed.

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or both hind limbs, and less often of one or both forelimbs, at times showed limpness of, and dragged their tails, occasionally had Jacksonian or generalized convulsions or head tremors, evidenced respiratory difficulty, became prostrated, and died

Methods—All of the mice used for pathological examination were killed by etherization in the period between the onset of paresis in one or more limbs and the time they became moribund. The autopsy was performed immediately after the cessation of respiration, the spinal cord and brain were removed first and fixed in either 10 or 20 per cent formalin solution. In some instances, segments of freshly removed spinal cord and brain were also fixed in formalin ammonium bromide solution, 95 per cent alcohol, and Zenker's solution. Occasionally the sciatic nerves and eyes were also removed and fixed in 10 or 20 per cent formalin solution. Specimens of each of the other organs were fixed in Zenker's solution in each instance. In fifteen mice, the brain and spinal cord were removed within their boney casings, the skull and vertebral column, and the whole fixed in 10 per cent formalin for 24 hours, the skull being perforated and the spinal canal being opened at a number of points. Decalcification was carried out in 1 per cent formic acid for 24 hours and the specimens sectioned for embedding. Paraffin sections cut at 4 to 5 micra were used for the majority of stains, at 10 to 18 micra for the Mahon stain, and frozen sections were cut for the metallic glial and fat stains.

As nearly as was possible, a complete sampling of the central nervous system was carried out, while only segments of the other organs, and occasionally sciatic nerves and eyes were examined. The appropriate stains for the demonstration of Nissl substance, myelin sheaths, neurofibrils, astrocytes, microglia, blood vessels, and other connective tissues and neutral fat were carried out on the neural tissues and the hematoxylin-eosin stain on the other organs.

In the study of the neural tissues, the findings in each animal were tabulated noting the position and approximate severity of the lesions. The intensity of the tissue reaction was graded roughly in the following fashion: + — — very slight, + — slight, + mild, ++ moderate, +++ marked, and ++++ very marked. The degree of local edema and congestion, the presence and degree of perivascular and diffuse parenchymal infiltration by hematogenous elements, the occurrence and extent of ganglion cell degeneration, the amount of microglial activation, the occurrence of astrocytic reaction, the presence of endothelial hyperplasia in the capillaries, and the degree of secondary leptomeningeal infiltration were all considered in determining the severity of the lesions.

Material—In all, fifty-six mice were studied in this fashion. Of these, nineteen had been inoculated intracerebrally, twelve intravenously, ten intraperitoneally, and five subcutaneously, while six had been infected by gavage and four by nasal instillation. In general, the pathological changes produced in the central nervous system upon inoculation of the virus by the various routes enumerated were similar at the late stage of the disease at which the animals were examined (between the initial paralysis and the moribund state).

GROSS APPEARANCE OF ORGANS—No clearly recognized gross abnormalities could be discerned in the brain or spinal cord, except for the occasional identification of the

inoculation tract in the intracerebrally infected animals. The spleen showed moderate enlargement in some of the animals, and the lungs were at times congested. Occasionally the liver was pale, and in one intravenously inoculated animal, it was yellowish in color.

HISTOLOGICAL FINDINGS —

Spinal Cord — The spinal cord was found to be the site of histopathological changes in fifty of the fifty-six mice. The six mice whose spinal cords proved to be negative will be discussed below. Of the fifty mice with positive findings, eighteen had been inoculated intracerebrally, twelve intravenously, nine intraperitoneally, and three subcutaneously, while four each had been infected by gavage and nasal instillation. The lumbar and sacral segments were involved forty-five times, the thoracic segments and the cervical region thirty-six times. One or both anterior horns showed abnormal changes at many levels of the spinal cord in each animal listed as positive, while the posterior horns were less often involved. Even in those mice in which the lesions were most severe, there were many levels at which no abnormalities were found. In addition to being the most frequent site of lesions, the lumbar cord was also the region where they were most severe. They were classified as 4+ in four animals, as 3+ in ten, as 2+ in ten, and as + in nine, while thirteen were + — and + — —. The cervical cord, although frequently involved, was less often implicated than the lumbar region. The majority of the lesions were approximately of the same severity as those at the lower level, but the most severe did not attain the intensity of the worst in the lumbar segments. Four mice showed 3+ lesions of cervical segments, ten 2+ lesions nine +, and thirteen + — or + — —. The changes in the thoracic region were relatively infrequent and also milder in degree. Only two mice showed 2+ lesions, while the other five had lesions of lesser severity.

Where the *anterior horns* were most severely affected, all of the grey matter was involved in its entire dorsoventral extent with some extension into the bases of the *posterior horns* (Fig 1). In the majority of instances, only a part of a given anterior horn showed abnormalities and these were usually in the ventral half of the horn. Where the lesions were least developed, they were at times confined to the ventral margin of the anterior horn.

The outstanding feature of the histopathology in the anterior horns was the degeneration of the *nerve cells* (Fig 1). On the average, one half or less of the ganglion cells were destroyed or were undergoing necrosis while the proportion ranged roughly from an involvement of one-tenth to a total disappearance of the neurocytes. The evidence of nerve cell injury ranged from mild changes, such as diffuse or central fragmentation of Nissl substance, to total disintegration of the cell (Fig 2). The chromatolysis was of varying degree and was accompanied by a swelling of the cell and often eccentricity or margination of the nucleus with final extrusion. Some cells had lost their Nissl substance, but had retained a centrally placed nucleus. The cytoplasm and nuclei of these cells showed varying degrees of loss of staining power, the increasing pallor being climaxed by complete fading out of the cell without fragmentation. The cytoplasm of other ganglion cells was coarsely or finely vacuolated or reticulated in addition to showing tigrolysis, and occasionally became coarsely granular. The nuclei at times showed irregular corrugation or crenation of their membranes with clumping of their chromatin. Occasionally the oxychromatin

became prominent or the nucleoli were eccentric or were extruded. Shrinkage of the nerve cell body was often associated with a total loss of Nissl substance, homogeneous cytoplasm which was deeply stained (eosinophilic in the hematoxylin-eosin stain), and shrunken central nucleus in which the chromatin material had completely coalesced into a solid, deeply staining oval mass in which the nucleolus could no longer be discerned. Intracellular neurofibrils in such heavily damaged necrotic cells were found to be extensively fragmented and the fragments swollen and distorted, and often neurofibrils could not be stained. In other nerve cells which gave evidence of lesser degrees of injury, the neurofibrils were often surprisingly well preserved, although coalescence, swelling, and tortuosity of the fibrils were occasionally seen. Swelling and distortion of dendrites, axones, and of myelin sheaths related to the degenerating ganglion cells were observed.

The *inflammatory reaction* was usually mild and relatively inconspicuous (Fig. 1). There was *perivascular infiltration* by lymphocytes, large mononuclear cells, and polymorphonuclear leucocytes. The cells in the Virchow-Robin spaces were not ordinarily numerous and often fewer in number than those located interstitially in the parenchyma of the involved anterior horn. In addition to being present in the affected grey matter, similar perivascular infiltration was encountered on radii, extending from the affected grey matter into the adjacent anterior and lateral white columns (Fig. 1). Occasionally near the point of emergence of an anterior, or entry of a posterior, root, a tiny focal area of edema and polymorphonuclear infiltration was encountered in the white matter. The *diffuse parenchymal infiltration* was somewhat more marked, and most often restricted to that portion of the anterior horn showing nerve cell degeneration (Fig. 1). Here lymphocytes and polymorphonuclear leucocytes were found near the damaged ganglion cells. Where the injury was obviously great and cell death had occurred, polymorphonuclear leucocytes were at times gathered at the cell membrane indenting it or lying in deep hollows in the cytoplasm. In some instances, the nerve cell, its nucleus gone and its cytoplasm granular, swarmed with polymorphonuclear leucocytes which permeated its disintegrating body (Figs. 2 and 3). A similar mild inflammatory reaction was observed in the *leptomeninges* in the ventral sulcus (Fig. 1), and over the ventral and ventrolateral aspects of the spinal cord, and rarely over its dorsolateral and dorsal surfaces. At times, it was very sparse or absent, and rarely it was moderate in degree. Lymphocytes, large mononuclear cells, and lesser numbers of polymorphonuclear leucocytes were present in the subarachnoid space and pia.

A moderate *microglial reaction* was present in the affected areas of the anterior horns in the majority of animals (Figs. 4 and 5), and in some instances, this was marked. In general, it paralleled the ganglion cell degeneration, lagging behind it. There might, however, be some dissociation so that the microglial activation outstripped the nerve cell destruction. The microglial cells became hypertrophied and multiplied. Their bodies became elongated, their processes shortened, coarsened, and irregular, and their nuclei lengthened and slender, and often curved or irregularly sinuous (Fig. 5). Conversion into lipid-laden phagocytes, so called compound granule or gutter cells, was rarely observed in this group of animals. The perineuronal proliferation of these elements about a degenerating nerve cell, known as satellitosis, was not common, although regularly observed. The same was true of their presence

in clusters within indentations and hollows in the cytoplasm of a necrotic nerve cell, participating in the process of neuronophagia

The *astrocytes* within a zone of degenerating nerve cells showed a minimal reaction. They became hypertrophied, their nuclei becoming more prominent, their cytoplasm increased in amount, and their processes were more numerous. This was always slight but was more frequent at the margins of the anterior horn near its junction with the white matter. Rarely the *capillaries* in an affected zone showed endothelial hyperplasia. The endothelial cells increased in number, their cytoplasm became more abundant and their nuclei plumper. Congestion was not common, and mild when present, and edema of the tissues even rarer. They were associated only with the severer lesions.

Reference has been made to six mice of the total of fifty six in which no cord lesions were found. The observations in only three of these are considered significant, the cord examination in the other three being incomplete. In view of the absence of lesions at many levels of the spinal cord in animals found to have lesions at other levels, the possibility is given that lesions were missed in these three.

Medulla—The medulla was involved in thirty five of the fifty six mice, fourteen having been inoculated intracerebrally, three intravenously, eight intraperitoneally, and five subcutaneously, while four were infected by gavage and one by nasal instillation. The lesions were of 4 + severity in one, 3 + in two, 2 + in eleven, + in thirteen, and + and + — in eight.

These were marked chiefly by foci of moderate perivascular and diffuse infiltration by lymphocytes and polymorphonuclear leucocytes, and by moderate microglial proliferation. Ganglion cell necrosis was rare and milder degrees of ganglion cell degeneration were common, but still relatively infrequent. Congestion, edema and capillary changes varied but were rarely conspicuous. Astrocytic reaction was virtually lacking except in the few severer lesions. The lesions occurred most frequently in the dorsal grey masses in the floor of the fourth ventricle and in the reticular formation. Leptomeningeal infiltration like that in the spinal leptomeninges was quite mild and occurred in the ventral midline and dorsolaterally, especially near the lateral angle of the fourth ventricle.

Pons—This portion of the brain stem was the site of lesions in thirty three mice, of whom, eleven were inoculated intracerebrally, seven intravenously, five intraperitoneally, and four subcutaneously, while five were infected by gavage and one by nasal instillation. The changes were for the most part moderate in degree and number. In one instance the lesions were 4 + in five, 3 + in twelve, 2 + in six, +, and in nine, + — and + — —. Here again, as in the medulla, lesions were most frequent in the grey matter in the floor of the fourth ventricle and in general in the dorsal half of the pons. They were often conspicuous at one lateral angle of the fourth ventricle and in the reticular substance. Their general nature was similar to that in the anterior horns with the difference that in general nerve cell degeneration although present, was not as conspicuous. In some foci it might be intense, but usually the diffuse infiltration by lymphocytes and polymorphonuclear leucocytes, the microglial activation and the perivascular infiltration overshadowed the ganglion cell changes. Congestion and edema varied as in the spinal cord and were usually relatively mild. Leptomeningeal infiltration by lymphocytes and

polymorphonuclear leucocytes occurred ventrally and near the lateral angles of the fourth ventricle

Cerebellum—This structure was involved in twenty-six mice. Eight were inoculated intracerebrally, seven intravenously, five intraperitoneally, and three each were infected by gavage and nasal instillation. The lesions were comparatively few in number and moderate in degree. In four mice, they were of 3 + intensity, in seven 2 +, in nine +, in six + - and + - -. They were located in the tectal nuclei and focally in the folia, involving the molecular and Purkinje cell layers (Fig 9). The histological character of the lesions was in general similar to that described in the other grey masses. Ganglion cell necrosis was seen somewhat more often than in corresponding lesions in the brain stem.

Midbrain—This portion of the brain stem contained lesions in nine mice. Five were inoculated intracerebrally, three intravenously, and one subcutaneously. The lesions were few and scattered, and were more often dorsal than ventral. The inflammatory changes, perivascular and leptomeningeal, usually predominated and the ganglion cell degeneration was rarely severe.

Hypothalamus—This area of the cerebrum was affected in eighteen mice. Six were inoculated intracerebrally, one intravenously, two intraperitoneally, and one subcutaneously, while five were infected by gavage and three by nasal instillation. The intensity of the changes were 4 + in one, 3 + in three, 2 + in three, + in four, and + or + - - in seven. They were thus for the most part mild and were moderate in number. Their histological character was similar to that of the midbrain lesions.

Thalamus—This locus was the site of lesions in thirty-three mice. Ten were inoculated intracerebrally, five intravenously, seven intraperitoneally, and three subcutaneously, while five were infected by gavage and three by nasal instillation. The lesions were 4 + in three mice, 3 + in five, 2 + in thirteen, + in eight, and + - and + - - in four. The majority of the lesions were, therefore, of moderate number in individual mice. These occurred more often in the dorsal than in the ventral portions of the thalamus and more frequently near the midline than laterally. The changes were predominantly diffuse and perivascular infiltration by cells like those seen elsewhere with associated microglial proliferation and relatively little, if any, ganglion cell degeneration. Such nerve cell changes were found principally in those intracerebrally inoculated animals in which the inoculation tract reached the thalamus.

Corpus Striatum—This zone was involved in twenty-two mice. Six each were inoculated intracerebrally and intravenously, two intraperitoneally, and three subcutaneously, while four were infected by gavage and one by nasal instillation. In six instances, the lesions were 3 +, in six 2 +, in six +, and in four + - in intensity. The changes varied, therefore, from mild to marked but were chiefly of the inflammatory and reactive microglial type with relatively few ganglion cell changes. Although occurring in many animals, lesions were few in any single animal and often unilateral.

Cerebral Cortex—The cerebrum was subdivided roughly into thirds in its rostro-caudal extent in recording the distribution of lesions. The changes in the olfactory centers, referred to below, were separately noted. Lesions of the pre- and post-

central areas of the anterior third of the parietal area and of the parorbital, anterior limbic, and prefrontal areas were listed under anterior third of the cerebrum lesions of the posterior two-thirds of the parietal area and of the superior temporal posterior limbic paracentral, and insular areas were listed as located in the middle third of the cerebrum, and lesions of the retrosplenial occipital, middle and inferior temporal, and calcarine areas as occurring in the posterior third of the cerebrum.

Anterior Third of Cerebrum—In spite of the fact that evidence of the inoculation tract, in the intracerebrally inoculated animals was encountered more than twice as frequently in the posterior third of the cerebrum as in the middle and anterior thirds combined the last was the most frequent site of severe lesions. The anterior third of the cerebrum was involved in thirty-one mice of which thirteen had been inoculated intracerebrally nine intravenously, four intraperitoneally, and one subcutaneously while two each had been infected by gavage and nasal instillation. In twelve the changes were of 4 + intensity in twelve 3 + in four 2 + and in three + or + - the average severity of the lesions being greater than that of those in the middle and posterior thirds of the cerebrum. The lesions were nearly always bilateral and more marked on one side. The ganglion cell changes the diffuse, perivascular, and leptomeningeal infiltration the microglial reaction and the other features of the pathological process were in general similar to those seen in the anterior horns of the spinal cord (Fig 6). Here again ganglion cell necrosis predominated, while the reactive inflammatory and glial changes were usually less intense and the leptomeningeal infiltration was secondary and focal.

Middle Third of Cerebrum—This area of the cerebrum was involved in twenty nine mice. Twelve of these animals had been inoculated intracerebrally six intravenously, four intraperitoneally and one subcutaneously while three each had been infected by gavage and nasal instillation. In three mice the lesions were of 4 + intensity, in eleven 3 +, in eleven 2 + and in four +. Although these lesions were fairly severe, on the average they were definitely less so than those in the anterior third of the cerebrum. They were ordinarily bilateral and usually more intense on one side. Their histological characteristics were like those described in the anterior third of the cerebrum.

Posterior Third of Cerebrum—This portion of the cerebrum was affected in twenty four mice, of which eleven were inoculated intracerebrally five intravenously, one intraperitoneally and two subcutaneously, while three were infected by gavage and two by nasal instillation. In ten of these mice the lesions were of 4 + intensity, in four 3 +, five 2 +, three +, and in two + and + -. The inoculation tract as noted above was very frequent in this area in the intracerebrally inoculated animals, and was associated with the severest lesions, predominating on one side. The histological features of the lesions were essentially similar to those seen elsewhere in the cerebrum often with the additional changes characteristic of a recent puncture wound of the brain.

Rhinencephalon—Under this designation are included ganglia olfactoria, tubercula olfactoria septal area, amygdaloid nuclei, and hippocampi. This region was involved in fifty-one mice of which sixteen had been inoculated intracerebrally, eleven intravenously eleven intraperitoneally, and three subcutaneously while six were infected by gavage and four by nasal instillation. Thirty mice showed lesions

of 4 + intensity, fourteen of 3 +, three of 2 +, two of +, and two of + — — The lesions were, therefore, very severe in the majority of the animals and this region was most regularly involved. The histological character of the changes was like that in other portions of the cerebrum with ganglion cell degeneration markedly predominating (Fig 7)

Olfactory Bulbs—Unfortunately, the olfactory bulbs were not examined in each mouse. They were studied in twenty-one mice of which two were inoculated intracerebrally, four intravenously, six intraperitoneally, and two subcutaneously, while three were infected by gavage and four by nasal instillation. Nineteen of these twenty-one mice had lesions in their olfactory bulbs. One of the intravenously, and one of the intraperitoneally inoculated animals were free of such changes. The lesions were of 4 + intensity in twelve animals, 3 + in three, 2 + in three, and + in one. The changes were of considerable severity, therefore, in the majority of the animals, including all of the intranasally infected, and some of each of those infected by each of the other routes. In three instances, in the two intracerebrally and one intraperitoneally inoculated mice, the lesions were isolated and unilateral. In most instances, however, the lesions were widespread and involved the glomerular, external granular, gelatinous, and mitral layers, and the external portion of the internal granular layer (Fig 8). When the changes were less severe, they affected only the outer layers, the glomerular and external granular laminae for instance or the internal granular layer alone. The nature of the histological abnormalities was similar to that described under rhinencephalon, the ganglion cell changes predominating.

Other Organs—The lungs were moderately congested in six of the fifty-six mice, the spleen showed congestion of its pulp and moderate numbers of polymorphonuclear leucocytes in its sinuses in ten, there were focal necroses of the liver in one mouse, and a fatty liver in another, an aspiration pneumonia in one, and acute gastric ulcers in one animal. These findings in other organs were obviously secondary or unrelated to the lesions in the central nervous system. No definite changes were detected in the lymphoid tissues that could be confidently related to the disease.

In reviewing the histopathological findings in the mice, it is clear that although a poliomyelitis of varying degree is constantly present, a polioencephalitis definitely overshadows it. The regular involvement of the anterior horns at either the lumbar or cervical enlargement or both is striking, but the average severity of the lesions and the frequency of involvement falls below that noted for the cortex in the anterior third of the cerebrum, in the rhinencephalon, and in the olfactory bulbs. As has been noted above, there are no outstanding differences in the distribution of the lesions at the late stages of the infection that can be correlated with the varying portal of entry. This will have to be investigated further in animals examined during preparalytic stages of the infection. The histopathological changes seen in the anterior horns are dominated by ganglion cell degeneration which on the average affects approximately half of the neurones, while the inflammatory reaction, perivascular, diffuse, and leptomeningeal and microglial activation are usually mild or moderate,

and congestion and edema are mild. The progressive diminution in ganglion cell involvement as one ascends the central nervous system is reversed in the cerebral cortex and olfactory centers and the relative degree of involvement or participation of the various elements in the process reverts to that observed in the anterior horns. The significance of this observation will be discussed later.

Observations on Guinea Pigs

Recently Jungeblut and Sanders (1941, 1942) (6, 8) succeeded in establishing the above murine virus in guinea pigs and in maintaining the infection in series. Transmission of the infection from mouse to guinea pig succeeded by intracerebral, intraperitoneal, intravenous, and subcutaneous routes, while serial passage from guinea pig to guinea pig is as yet successful only by intracerebral inoculation. Single passages from guinea pig to guinea pig succeeded a few times by intravenous inoculation of brain and spinal cord. The effective dose of the murine virus for intracerebral inoculation of guinea pigs was $\frac{1}{10}$ cc. of a 10 per cent suspension of mouse brain,¹ while intraperitoneally, subcutaneously, and intravenously, 1 to 2 cc were necessary. In transmission from guinea pig to guinea pig, by the intracerebral route, $\frac{1}{10}$ cc¹ of a 10 per cent suspension of mixed brain and spinal cord was effective, while the few times that intravenous inoculation succeeded 1 cc was used. The incubation period was approximately 2 to 4 days. A preparalytic fever terminated when flaccid paresis of the hind limbs supervened. This was sometimes more marked on one side. There was rapid progression to complete flaccid paralysis of the hind limbs, while the animal remained alert and apparently well otherwise. Its fur remained sleek and it often moved along briskly by the active use of its forelimbs while the hind limbs dragged behind. Weakness of one or both forelimbs at times followed, and occasionally there was slight intermittent tremor of the head. Complete flaccid paralysis of one or both forelimbs might develop, respiratory difficulty occurred, and the animal became prostrated and died in from 5 to 8 days. More recently, the course of the infection in serial guinea pig passages became more rapid so that the animals died in from 3 to 5 days. Partially paralyzed animals have recovered.

Methods—The guinea pigs were killed by chloroform and their brains, spinal cords, and other organs were removed at once and fixed in 10 per cent formalin. The subsequent handling of the tissues was similar to that employed for the mice. In the study of the neural lesions, their approximate position and severity was noted and the intensity graded in the same manner as described for the mice.

Material.—A group of sixteen guinea pigs were studied histologically. Of these,

¹On occasions, dilutions of 1:500 have been effective.

eight were inoculated intracerebrally with $\frac{1}{10}$ cc of a 10 per cent mouse brain suspension of the 72nd or a subsequent serial passage of murine poliomyelitis virus, one was inoculated intraperitoneally with 2 cc of a similar 10 per cent mouse brain suspension, one was inoculated subcutaneously with 2 cc of a 10 per cent mouse brain suspension, four were inoculated intracerebrally with $\frac{1}{10}$ cc. of a 10 per cent suspension of mixed guinea pig brain and spinal cord, and two were inoculated intravenously with 1 cc of a similar suspension. The animals were sacrificed between the time of development of a complete flaccid paralysis of the hind limbs and the occurrence of prostration.

In this late stage of the disease, the pathological findings were in general similar, except for the supervention of a separate, clearly distinguishable additional toxoplasmic infection in the two guinea pigs which had been inoculated intravenously with a cavian strain. Their spinal cord and some of their brain stem lesions were in all respects similar to those to be described below in the remaining fourteen guinea pigs and clearly due to the transmitted virus. These two animals will be reported upon separately elsewhere.

GROSS FINDINGS—Except for evidence of the inoculation tract, no gross abnormalities were noted in the brain or in the spinal cord. The spleen was slightly enlarged in three animals. The lungs were mildly congested in three guinea pigs and considerably so in three. In the last group of animals, there were small, slightly raised, lighter colored areas on the cut surfaces of the lower lobes.

HISTOLOGICAL FINDINGS—

Spinal Cord—The spinal cord was the site of lesions in all *fourteen* guinea pigs. The *lumbar* and *sacral* segments were involved in each, the anterior horns always being affected, while the posterior horns showed abnormal changes in all but one. The intensity of the lesions in the anterior horns in this region was 4 + in ten animals, 3 + in three, and 2 + in one, while in the posterior horns, they were considerably less marked, being 4 + in one, 3 + in two, 2 + in five, + in two, and + — and + — — in three.

The *thoracic* segments were involved in nine guinea pigs, the anterior horns being affected in each, and the posterior horns in five. The intensity of the lesions in the anterior horns was 4 + in four, 3 + in two, and 2 + in two, while in the posterior horns, it was 4 + in two, 3 + in one, and + in two. Thus, although the abnormal changes were less frequent in the thoracic region, they were of the same order of severity as in the lumbosacral segments. The posterior horns were relatively less frequently involved in this region and again the lesions were less intense on the average than in the anterior horns.

The *cervical* segments were involved in all fourteen guinea pigs, the anterior horns being affected in each and the posterior horns in twelve. The severity of the changes in the anterior horns was 4 + in ten animals, 3 + in three, and + — — in one, while in the posterior horns it was 4 + in three animals, 2 + in five, + in two, and + — — in two. The lesions were, therefore, similar in frequency, distribution and intensity in this region to those in the lumbosacral portion of the spinal cord.

The *anterior horns* showed abnormal changes throughout their entire dorsoventral extent where the lesions were most severe (Figs 10, 11, and 13) and at corresponding levels the *posterior horns* might exhibit equally intense changes throughout or in their

ventral two-thirds. It was more often the case that the ventral half or less of the given posterior horn was affected, and that the lesions were much less intense. Where the anterior horns were only partially affected, only the ventral extremities of the posterior horns might be involved or they might be free of lesions. At some levels only one anterior horn was involved (Fig 10).

The most conspicuous feature of the process in the anterior horns was the degeneration of the *nerve cells* (Figs 10, 13, 14, and 17), and where this was most severe they might completely disappear (Fig 13). More often from one half to two-thirds of the neural elements were found in varying stages of degeneration, while the remainder might be remarkably well preserved. The details of the ganglion cell changes were essentially like those observed in the anterior horns of the spinal cord in the mice, and, therefore will not be described further. Necrosis of ganglion cells occurred as well in the posterior horns, chiefly in their ventral portions, but was much less frequent there. In one guinea pig of those inoculated intracerebrally single or multiple round eosinophilic intranuclear bodies somewhat smaller than the nucleolus were found in some of the ganglion cells (Fig 18). There was no margination of the chromatin.

Although the *inflammatory reaction* was moderate, it was definitely more advanced than in the mice. Both the *perivascular* and *diffuse infiltration* were more florid (Fig 13) the latter more so than the former. Lymphocytes, large mononuclear cells, and polymorphonuclear leucocytes were present in the perivascular spaces (Fig 21) in variable numbers in the anterior horns and in the affected portions of the posterior horns and in the latter they might overshadow the ganglion cell changes. Similar perivascular infiltration was found irregularly and to a minor degree in the white columns near the involved portions of the grey. The diffuse infiltration consisted of lymphocytes and polymorphonuclear leucocytes (Figs. 14, 16, and 17), and more closely paralleled but always lagged behind the nerve cell changes. Polymorphonuclear phagocytosis of nerve cells similar to that described in the mice was frequent (Figs. 15 and 16). *Leptomeningeal infiltration* was quite mild and was usually ventral, including the ventral sulcus (Fig 12). In places, it was also seen in the dorsal and dorsolateral portions of the leptomeninges as well. It was chiefly lymphocytic in type (Fig 20) with variable numbers of polymorphonuclear leucocytes and large mononuclear cells.

The *microglial reaction* was similar in type to that observed in the mice but was in general much more marked in degree (Fig 17). It always paralleled the ganglion cell degeneration and at times passed beyond it in intensity. In such instances, the cellularity of the involved anterior horns was greatly increased the welter of lymphocytes, polymorphonuclear leucocytes and hypertrophied microglial cells being dominated by the latter. Where the changes were severest, there occurred a moderate conversion of microglia into lipid laden phagocytes, or compound granule cells. This was more frequent and more conspicuous than in the mice, but never massive. These phagocytes were encountered in the perivascular spaces in varying numbers in such zones. Glial satellitosis and neuronophagia was often observed (Fig 17).

Astrocytes exhibited the same minimal reaction described in the mice. Where the lesions were severest, moderate *congestion* and considerable *edema* might be observed

(Fig 13) and these were on the average greater than was usually seen in the mice. Occasional small recent hemorrhages were encountered in some of the more intensely involved anterior and posterior horns. Mild endothelial hyperplasia of the capillaries was seen with a fair degree of regularity in the affected grey matter.

Medulla—The medulla was the site of lesions in two of the fourteen guinea pigs. These were small, scattered and few in number. They were marked chiefly by perivascular and, at times, associated focal leptomeningeal infiltration by lymphocytes, and less frequently, fewer large mononuclear cells and polymorphonuclear leucocytes. Rarely similar cells were seen in the nearby parenchyma. Ganglion cell degeneration and microglial activation were lacking. In two additional guinea pigs, there were mild focal areas of leptomeningeal infiltration ventrally and laterally without the presence of parenchymal lesions.

Pons—In two guinea pigs, moderate to marked focal lesions were encountered, chiefly in the dorsal portion of the tegmentum. Their essential histological features were similar to those of the changes observed in the anterior horns with the difference that ganglion cell degeneration was not nearly as prominent. There was mild ventral and dorsal ventrolateral infiltration of the leptomeninges of the same character seen about the medulla.

Cerebellum—None of this group of fourteen guinea pigs showed any changes in the cerebellum.

Midbrain—Three guinea pigs exhibited lesions in this portion of the brain stem. These were few in number, mild in degree, bilateral, chiefly dorsal, and of the same character as in the medulla. Perivascular infiltration by lymphocytes was their chief characteristic and when they were near the surface there was an associated focal leptomeningeal area of infiltration. In four additional guinea pigs, mild or marked leptomeningeal infiltration by lymphocytes and occasional large mononuclear cells and polymorphonuclear leucocytes were seen on the ventral surface of the midbrain.

Thalamus—Four guinea pigs showed scattered and limited lesions in the anterior and posterior portions of the dorsal aspect of the thalamus near the midline. These were unilateral in three animals, and associated with the inoculation tract in one. Three were of + intensity and one was 2+. Ganglion cell degeneration, except as associated with the inoculation tract, was absent. Mild perivascular and diffuse infiltration by lymphocytes and occasionally polymorphonuclear leucocytes and a somewhat more marked microglial proliferation were their essential features.

Hypothalamus—No strictly hypothalamic lesions were encountered in this group of guinea pigs.

Corpus Striatum—This portion of the cerebrum showed limited involvement in three animals. In two, the lesions were associated with a portion of the inoculation tract, were unilateral, and were of 4+ and + severity respectively, while in the third they were of + intensity. Their character was similar to that of the lesions described in the thalamus.

Cerebral Cortex—The same rough subdivision into thirds in recording the distribution of lesions described for the mice was employed for the guinea pigs.

Anterior Third of Cerebrum—The cortical areas in this portion of the cerebrum contained lesions (Fig 19) in five guinea pigs. These were of 4+ intensity in two,

3 + in one. They were associated with an inoculation tract in three animals and were relatively few in number. In two, the lesions were bilateral. Ganglion cell degeneration was present in these lesions, but was usually mild. In a few, it was severe. The accompanying inflammatory reaction consisted of moderate and sometimes marked perivascular and diffuse infiltration by lymphocytes and, at times, polymorphonuclear leucocytes. The microglia hypertrophied and multiplied, and this change was at times quite marked.

Middle Third of Cerebrum—The cortical areas in this zone were affected in seven guinea pigs. Three showed lesions of 3 + intensity one of 2 +, one of +, and two of + —. In three the lesions were bilateral and in four they were associated with the inoculation tract. In general, they were even fewer in number and of lesser severity than those in the anterior third of the cerebral cortex but had similar histological features.

Posterior Third of Cerebrum—This zone of the cortex was involved in six animals which showed lesions of 4 + intensity in three and + — — severity in the three others. The lesions were all unilateral and associated with an inoculation tract in three guinea pigs. They were limited in number and extent and in the three instances in which they were very mild, consisted essentially of slight leptomeningeal and nearby cortical perivascular infiltration by lymphocytes. The severer lesions in the remaining three animals resembled the more intense, but limited changes described in the anterior third of the cerebrum.

Rhinencephalon—Lesions were present in seven animals all intracerebrally inoculated. In two, these were confined to the hippocampi. Four had only unilateral lesions. Three of these showed lesions of 4 + intensity and were associated with the inoculation tract. Of the remainder, one had lesions of + severity one of + — and three of + — —. Except in the severest lesions, ganglion cell degeneration was absent or minimal and the changes were inflammatory and glial.

Olfactory Bulbs—The olfactory bulbs were examined in nine guinea pigs. They contained no lesions in seven animals, unilateral isolated 4 + lesions in one animal, and a few unilateral 2 + lesions in another. In each of the affected animals the lesions were near the posterior extremity of the bulb and the gelatinous, mitral, and internal granular layers were involved, particularly the last. Perivascular and diffuse infiltration by lymphocytes and microglial reaction were associated with little or no ganglion cell degeneration.

Other Organs—The spleen showed congestion of the pulp and moderate numbers of polymorphonuclear leucocytes in the sinuses in three guinea pigs. In three animals there was mild pulmonary congestion and in three others this was severe and associated with a lobular pneumonia. No lesions were found in the kidneys.

A review of the histopathological findings in the central nervous system in the guinea pigs leads to the conclusion that one is dealing with an intense poliomyelitis with which is associated a minor degree of meningo-encephalitis. The lumbar and cervical enlargements of the spinal cord are markedly involved in every animal, the anterior horns being the site of the severest lesions, while the posterior horns are frequently but moderately affected. Ganglion cell degeneration is the chief feature of the

process, often involving one-half to two-thirds of the neurones, and not infrequently all of them. The inflammatory reaction, perivascular and diffuse, is mild or moderate in degree and the associated leptomeningeal infiltration is similar in type and limited in extent and location. Congestion and edema are usually mild or moderate, but they may become quite intense, particularly in the latter, and may be associated with small, fresh hemorrhages. Microglial activation is usually marked and at times very extensive. In general, there is a progressive diminution in the severity and number of the lesions as one ascends the central nervous system. Ganglion cell degeneration becomes progressively less prominent as one passes cephalad and the changes are essentially inflammatory and glial. The increase in the frequency and severity of the lesions in the cerebrum as compared to the brain stem is associated with, and principally confined to, the area of inoculation. The olfactory bulbs show only isolated lesions in two of the nine animals in which they were examined. In contrast to the mice, the cerebellum is not involved.

DISCUSSION

Whether the virus of human poliomyelitis has been transmitted from the monkey to rodents cannot be decided solely from a study of the pathologic changes in the central nervous system of the latter. One difficulty is the possibility that different species may react in different ways to the same virus. Another is that the types of reaction of the neural, neuroglial, and their associated mesodermal structures are rather limited and hardly specific for more than a very few conditions. Only through the biological evidence can a final conclusion be reached. However, with these reservations, the histology and localization of the lesions can offer corroborative evidence for the identity of the rodent disease with human poliomyelitis.

Comparison of Lesions Induced in Mice by the J-S, Armstrong, and Theiler Viruses

In the *mouse*, infected under the conditions herein described, one is dealing with a meningoencephalomyelitis. The encephalitis, particularly in the anterior third of the cerebrum, including the olfactory centers, definitely outweighs the myelitis in extent and gravity. The latter, however, is distinctly marked by being a poliomyelitis with preferential localization in the anterior horns. A comparison of the findings in these mice with those in the mice reported as infected with the Lansing strain of poliomyelitis by Armstrong (1, 2), and with those having Theiler's disease as reported by Theiler (11) and by Olitsky and Schlesinger (12) reveals certain similarities and a number of differences. Common to all is a meningoencephalomyelitis in which lesions of the anterior horns are a fairly constant feature, though often mild or moderate in degree. The details of the histopathological process in the anterior horns are essentially similar in each. Ganglion cell degeneration is the outstanding feature. Differences in the degree of inflammatory reaction, microglial

activation, and capillary proliferation probably depend upon the differences in rapidity of development of the process. The J-S virus is quite virulent and infection with it leads to death in 2 to 4 days, the Armstrong virus is less virulent, and mice often live longer than a week while most strains of the Theiler virus do not produce paralysis till 4 days or more after inoculation. The absence of lesions in many segments of the spinal cord is noted both in the Armstrong and the present Jungeblut Sanders group of animals. The spinal cord lesions are more frequent at the cervical level in the Armstrong group, and at the lumbar level in the J-S mice. Polymorphonuclear leucocytes are, perhaps more frequent in the anterior horn lesions in the J-S animals than in those of either of the other two groups, and neuronophagia possibly somewhat more common in this group and the Armstrong group than in the Theiler mice.

The brain stem shows lesions in approximately three fifths of the Jungeblut Sanders mice, but the degree of ganglion cell degeneration is much less than that in the anterior horns of the spinal cord. The medulla and pons are about equally involved, the dorsal areas and reticular formation most commonly. The midbrain is relatively infrequently affected. In the Armstrong mice, the brain stem is involved in nearly all the animals and the lesions show a similar distribution. This difference in frequency may again be dependent upon the more rapid course of the infection with the J-S virus. In general, the brain stem distribution of the lesions in the Theiler mice as described by Olitsky and Schlesinger (12) follows the same pattern given for the other two and the histological changes are of similar type in all three with neuronal degeneration being present but not prominent.

In the J-S mice the cerebellum is involved in approximately half the animals, both the tectal nuclei and cerebellar cortex being affected. The lesions are moderate in degree and include somewhat more ganglion cell degeneration than do those of the brain stem. This is in contrast to the Armstrong and Theiler mice in which the cerebellar lesions are almost wholly of the tectal nuclei and milder in degree, chiefly inflammatory and reactive glial.

The hypothalamus is the site of lesions in over one third of the J-S mice. Perivascular infiltration and microglial activation predominates in them. This area is not separately referred to in the other two series of mice.

The thalamus is affected in approximately three fifths, and the corpus striatum in approximately two-fifths of the J-S mice. The lesions are essentially like those in the hypothalamus, and less frequent in the corpus striatum than in the thalamus where they are most often dorsal and near the midline. Ganglion cell degeneration is usually absent or mild except where the inoculation tract enters the area involved. Thalamic and striatal lesions are of the same type in the Armstrong and Theiler mice (Olitsky and Schlesinger) but much less frequent.

The cerebral neocortex is severely and extensively involved in three-fifths of the J-S mice and the anterior third of the cerebrum is most often hit. Ganglion cell degeneration again becomes quite prominent in this portion of the central nervous system in contrast to the findings in the central and basal nuclei of the cerebrum, and in the brain stem. In the Theiler mice (Olitsky and Schlesinger), such cortical lesions are present in almost all the animals but neuronal degeneration is apparently much less prominent and the lesions are very much fewer. The cortex is affected in

less than half of the Armstrong mice, and the lesions are again described as infrequent and the nerve cell degeneration as minimal

The rhinencephalon, including the hippocampi, is affected in almost all of the J-S mice, and to a marked degree. The severity of the changes resembles that in the neocortex and often exceeds it. The hippocampi are described as being the structures in the cerebrum most frequently involved in the Armstrong mice.

The olfactory bulbs are regularly and often severely involved in the majority of the J-S animals inoculated by all the routes listed. No such lesions are encountered in the Theiler mice studied by Olitsky and Schlesinger, even in those infected by intranasal instillation and responding by paralysis. No data on this point are available in the Armstrong series.

In summary, the pathological changes in the central nervous system of the J-S, Armstrong, and Theiler mice are alike in that lesions of the anterior horns are fairly constant and similar in type in each, that each is marked by a meningoencephalomyelitis and that as one passes cephalad in the central nervous system, the frequency of ganglion cell degeneration progressively diminished in all (at least up to the cerebral cortex and excepting the cerebellum). They differ in that the J-S mice show much more severe and extensive involvement of the neocortex, rhinencephalon, and to a lesser degree of the cerebellum, while marked lesions are present in the olfactory bulbs as compared to a total lack of them in the Theiler mice. The greater virulence of the J-S virus may be in part responsible for these differences so that with a breakdown of local tissue immunity, the given area is overwhelmed. This is, however, not in consonance with the finding of approximately equal lesions in the anterior horns of the spinal cord in all three series of mice. The question of any similarity between the meningoencephalomyelitis in these mice and the meningoencephalomyelitis of monkey and man which goes by the name of poliomyelitis will be discussed later when the same problem arises in relation to the pathological changes in the central nervous system of the guinea pig.

In spite of the histopathological similarities between the disease in the J-S mice and those having Theiler's disease, it is unlikely that the latter spontaneous disease of mice has been induced in the Jungeblut-Sanders animals. The differences in virulence of the two viruses, when tested in young mice by peripheral routes, or in old mice by intracerebral injection, together with the fact that normal adult mouse serum neutralizes Theiler's virus but not the J-S virus, bar such a conclusion.

Comparison of Lesions Induced in Guinea Pigs by the J-S Virus with Those in So Called Spontaneous Guinea Pig Poliomyelitis

Roemer and Joseph (13) (1910) described a presumably spontaneous flaccid paralysis in 5 per cent of their guinea pig stock, and later Roemer (14) (1911) was able to

isolate a virus from these animals but this was lost after a few passages. The latter pointed out what he considered to be the similarity of the clinical features of this disease to those of human poliomyelitis. He summed up the pathological changes as "meningo-myo-encephalitis of predominantly lymphocytic type. The dominating change was the meningitis which his illustration of the spinal cord shows to have been of considerable intensity. The exudate was composed of lymphocytes with some polymorphonuclear leucocytes. Perivascular infiltration of the same sort was seen in the white matter, while the grey matter also showed diffuse infiltration which was particularly marked about the central canal. The anterior horns were less involved. The ganglion cells were well preserved at first but with the onset of paralysis some disappeared and others stained poorly. Neuronophagia was rare. The lumbar cord was always more intensely involved than the thoracic or cervical regions. The medulla was described as being only slightly affected. The cerebral leptomeninges both dorsal and ventral, were heavily infiltrated, chiefly by lymphocytes, the ventricles contained a similar exudate and there was marked perivascular lymphocytic infiltration in the underlying cortex. The author concluded that there was a similarity between the pathologic changes in these guinea pigs and those of human poliomyelitis. This does not appear to be borne out by the facts he has recorded.

Neustaedter (15) (1913) and Picard (16) (1925) reported the occurrence of what they interpreted as spontaneous poliomyelitis in guinea pigs which had been in close proximity to poliomyelitis-infected monkeys. These animals again showed predominantly flaccid paralysis of the hind limbs. The virus obtained from each group of animals was lost after a few passages. An emulsion of spinal cord and in one instance material from the nasal mucosa was used for intranasal instillation in guinea pigs by Neustaedter who also claimed to have successfully transmitted the infection from a monkey to a guinea pig and back to a second monkey by nasal instillation of filtered cord material from the first monkey to the guinea pig and inoculation of an emulsion of the brain and cord of this guinea pig into the second monkey. Picard inoculated an emulsion of spinal cord of one of his guinea pigs showing spontaneous flaccid paralysis of the hind limbs intraperitoneally into other guinea pigs with the production of a similar picture. Neustaedter briefly described the pathologic changes in his guinea pigs as consisting of mononuclear infiltration of the leptomeninges in the ventral sulcus pericellular infiltration by similar cells in the anterior horns advanced degeneration of anterior horn ganglion cells neuronophagia, and hemorrhages. Pial infiltration by lymphocytes was noted about the olfactory bulbs in one of the four guinea pigs examined and in one other, the brain was examined and reported free of lesions. Picard recorded the pathologic findings in one of his guinea pigs in the following abbreviated form: "only mild infiltration of the blood vessels, scattered hemorrhages in the anterior horns and no definite edema. Severe degeneration of the nerve cells vacuolization, pallor and swelling particularly in the motor elements of the anterior horns. Neuronophagia by glial cells is relatively slight although occasionally seen." No mention was made of the rest of the central nervous system.

In the guinea pigs inoculated with the J-S murine virus or subinoculated with theavian-passage virus, the histopathological changes in the central nervous system

are marked by a predominant involvement of the spinal cord. The anterior horns are primarily affected and in them the degeneration of nerve cells is the outstanding phenomenon. One-half to two-thirds or more of the neurones are affected and this involvement is definitely more marked than in the mice. The associated inflammatory and microglial reactions, particularly the latter are more prominent than in the mouse, and congestion and edema are more conspicuous. There is a striking decrease in the intensity and frequency of lesions in ascending levels of the central nervous system and ganglion cell degeneration diminishes in the same order. A detailed comparison with the distribution of lesions in the brain in human and simian poliomyelitis, as recorded by Bodian and Howe (17) and others, cannot be made until a study of preparalytic stages of the infection in guinea pigs has been carried out.

The pathologic changes in the Roemer animals are difficult to evaluate and compare. In spite of the paucity of recorded findings, the changes in the spinal cords of the Neustaedter and Picard guinea pigs show broad resemblances to those in the J-S animals.

Pathologic Evidence Bearing upon the Identity of the J-S Virus

The investigations of Jungeblut and Sanders lead them to believe that they have transmitted the SK New Haven strain of poliomyelitis virus from monkey to cotton rat to mouse and then to guinea pigs. The histopathologic changes in their mice do not in themselves substantiate this conclusion inasmuch as the rather constant but mild anterior poliomyelitis is accompanied by a severe encephalitis. The anterior poliomyelitis, however, is striking. In spite of the fact that a severe poliomyelitis, in the sense of a marked degeneration of neurones with associated inflammation and glial reaction in the grey matter of the spinal cord, may occur in mice in certain other experimental virus infections, such as louping ill, vesicular stomatitis, equine encephalomyelitis, and St. Louis encephalitis, there are features which distinguish these from the J-S infection.

While the anterior horns of the spinal cord are regularly involved in mice infected by the J-S virus no matter what the portal of entry, they are affected in vesicular stomatitis only if the portal be a peripheral one, such as muscle. In louping ill, the constancy of the involvement of the Purkinje cells, which are only secondarily and focally affected in the J-S animals, is one of a number of differential points. In equine encephalomyelitis in mice, the rhinencephalic cortex and hippocampus are severely affected as they are in the J-S mice, but the occurrence of intranuclear inclusions in the nerve cells in the former serves to distinguish between them. The greater degree of leptomeningeal infiltration and often of perivascular infiltration as well, and the frequency of the perivascular localization of lesions in the mice with St. Louis encephalitis are differential points.

It is interesting that the anterior third of the cerebrum, in which the motor area resides, is most frequently hit by the J-S virus, although in the intracere-

brally inoculated animals, the middle and posterior thirds were most often injected. This reminds one of the frequency of involvement of the motor area in poliomyelitis of monkey and man. On the other hand, the constancy of involvement of the olfactory bulbs in the J S mice, no matter what the portal of entry be, is quite unlike the lack of lesions in these structures in human and simian poliomyelitis. In brief, there is no compelling evidence in the pathological picture in these mice which would lead one to conclude that they were suffering from infection with the virus of human poliomyelitis.

This is not true, however, of the lesions which result from transfer of the murine virus to guinea pigs. In the guinea pig, the changes in the central nervous system are remarkably like those of poliomyelitis of monkey and man. This similarity persists through repeated transmissions from mouse to guinea pig, and in the cavian series of subinoculations. It is most interesting that this species change in the character of the lesions in guinea pigs is attended by a marked fall in virulence of the virus and occasional transmissibility to monkeys. While dilutions of virus of a million or more are effective when inoculated intracerebrally in mice, dilutions approximating those necessary for the infection of monkeys by the virus of poliomyelitis are necessary in guinea pigs. The distribution and histological details of the lesions in the guinea pig approach those of the simian and human disease, poliomyelitis, very nearly. This is in accord with evidence submitted by Jungeblut and Sanders indicating that the SK strain of human poliomyelitis has actually been transferred to rodents.

SUMMARY

A description has been given of the lesions produced in mice and guinea pigs by inoculation of the Jungeblut-Sanders virus. The histopathological findings, although in themselves not conclusive, would tend to support the opinion that Jungeblut and Sanders have transmitted the SK poliomyelitis virus to mouse and guinea pig. In mice the virus apparently retains its affinity for the anterior horns of the spinal cord, but in a moderate degree. Associated with a marked increase in virulence of the virus, a strong affinity for the cerebral tissues, more particularly the olfactory centers, develops. On transmitting this murine variant of the virus to guinea pigs, however, the original character of the virus is again revealed. There is a reversion to a predominant affinity for the nerve cells of the anterior horns of the spinal cord.

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EXPLANATION OF PLATES

PLATE 1

FIG 1 *Mouse 9 Spinal cord* Almost total disappearance of ganglion cells in anterior horn of lumbar enlargement Diffuse infiltration of grey matter by lymphocytes and polymorphonuclear leucocytes and mild perivascular infiltration Considerable microglial proliferation Moderate leptomeningeal infiltration, ventrolaterally and in ventral sulcus Hematoxylin-eosin stain $\times 110$

FIGS 2 and 3 Fig 2, *mouse 27*, Fig 3, *mouse 33 Spinal cord* Necrotic nerve cells in anterior horns of lumbar enlargement undergoing polymorphonuclear phagocytosis Note pericapillary polymorphonuclear leucocytes and lymphocytes in Fig 3 and similar mild diffuse infiltration in both Figs 2 and 3 Hematoxylin-eosin stain $\times 460$

FIG 4 *Mouse 3 Spinal cord* Early microglial proliferation in area of neuronal degeneration in anterior horn of lumbar segment Hortega's silver carbonate stain $\times 460$

FIG 5 *Mouse 3 Spinal cord* Advanced reactive hypertrophy of microglia in zone of nerve cell necrosis in anterior horn of lumbar segment Hortega's silver carbonate stain $\times 460$



PLATE 2

FIG 6 *Mouse 17 Cerebrum—anterior third* Focus of necrosis of cortical ganglion cells with neuronophagia. Mild diffuse and leptomeningeal, and mild perivascular lymphocytic and polymorphonuclear infiltration. Hematoxylin-eosin stain

FIG 7 *Mouse 37 Cerebrum* Similar lesion to that in Fig 6 with less inflammatory reaction in the hippocampus. Hematoxylin eosin stain

FIG 8 *Mouse 59 Olfactory bulb* Perivascular and diffuse infiltration by lymphocytes in internal granular, mitral, gelatinous, external granular, and glomerular layers and capsule. Loss of nerve cells in all the affected layers. Hematoxylin-eosin stain

FIG 9 *Mouse 8 Cerebellum* Folium showing focal loss of Purkinje cells, diffuse and leptomeningeal lymphocytic infiltration, and a considerable microglial proliferation. Hematoxylin eosin stain



PLATE 3

FIG 10 *Guinea pig 5 Spinal cord Cervical segment* Total loss of ganglion cells in one anterior horn Intense polymorphonuclear and lymphocytic infiltration and microglial proliferation Moderate extension of inflammatory and reactive glial process into posterior horns Hematoxylin eosin stain

FIG 11 *Guinea pig 9 Spinal cord Cervical segment* Almost complete loss of neurones in one anterior horn Lymphocytic and polymorphonuclear infiltration, microglial proliferation, and edema Slight perivascular and leptomeningeal infiltration (ventral sulcus) Hematoxylin eosin stain

FIG 12 *Guinea pig 9 Spinal cord—Cervical segment* Changes in anterior horn like those in Fig 11 Note mild infiltration of ventral leptomeninges and those in ventral sulcus by round cells Hematoxylin eosin stain

FIG 13 *Guinea pig 9 Spinal cord Lumbar segment* Extensive nerve cell degeneration in both anterior horns Inflammatory and microglial reactions, intense and more marked on one side Considerable edema Mild infiltration of leptomeninges in ventral sulcus

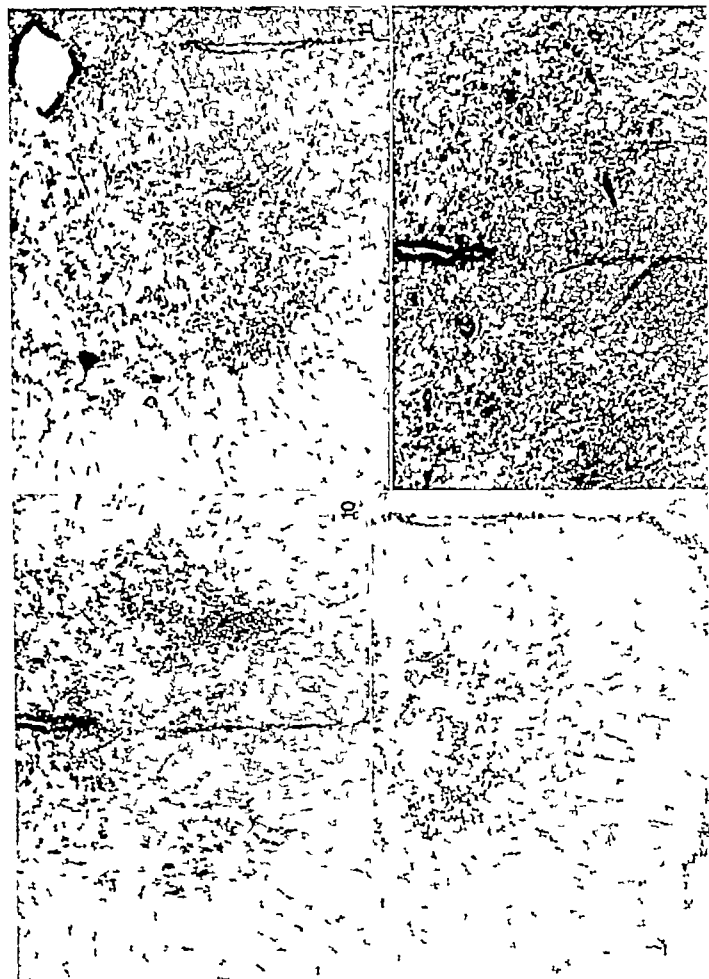


PLATE 4

FIG 14 *Guinea pig 9 Spinal cord* Anterior horn of *cervical* segment Nerve cells in various stages of degeneration and polymorphonuclear phagocytosis Diffuse infiltration by polymorphonuclear leucocytes and lymphocytes Mild perivascular round cell infiltration Hematoxylin-eosin stain $\times 230$

FIG 15 *Guinea pig 5 Spinal cord* Anterior horn of *lumbar* segment Degenerated nerve cells undergoing early polymorphonuclear phagocytosis Note well preserved nearby neurone Mild diffuse polymorphonuclear and lymphocytic infiltration and slight perivascular round cell infiltration Hematoxylin-eosin stain $\times 460$

FIG 16 *Guinea pig 9 Spinal cord* Anterior horn of *cervical* segment Detail of Fig 14 Note advanced polymorphonuclear phagocytosis of necrotic nerve cell and partially preserved nearby ganglion cell Polymorphonuclear leucocytes and lymphocytes in surrounding tissues Hematoxylin eosin stain $\times 460$

FIG 17 *Guinea pig 5 Spinal cord* Anterior horn of *lumbar* segment Degenerating nerve cell showing satellitosis Diffuse lymphocytic and polymorphonuclear infiltration and advanced microglial proliferation and early neurophagia Hematoxylin-eosin stain $\times 460$

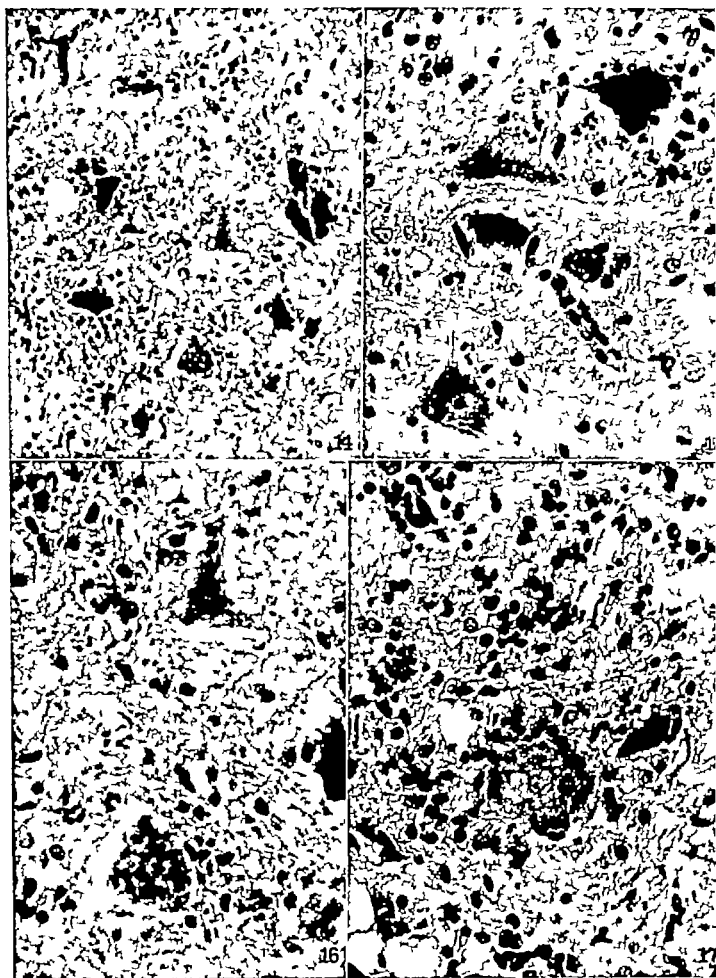


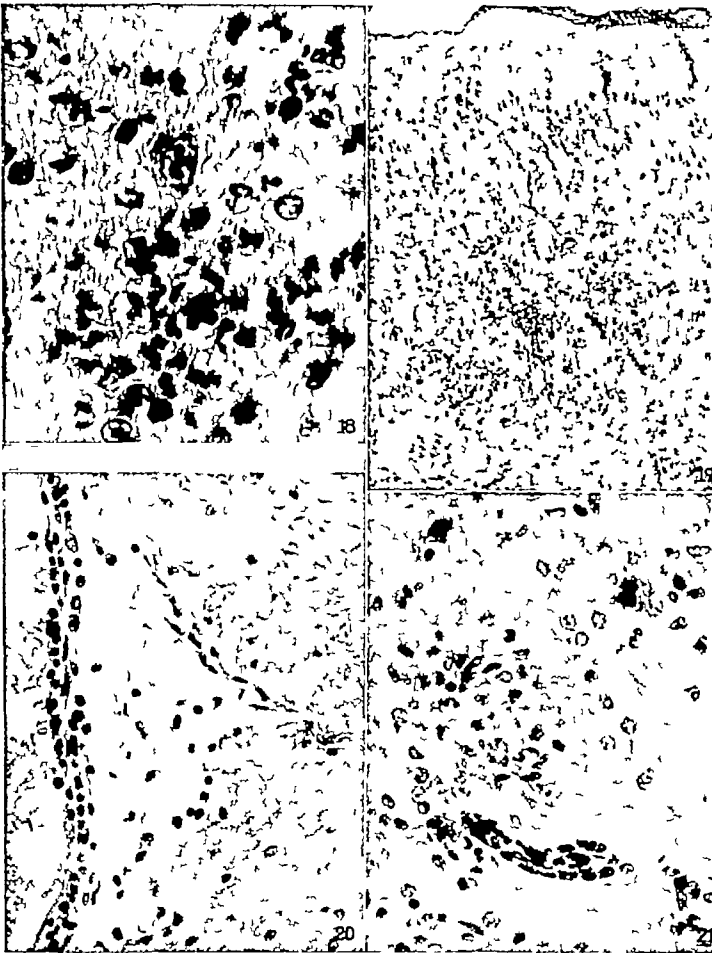
PLATE 5

FIG 18 *Guinea pig 4 Spinal cord* Anterior horn of lumbar segment Eosinophilic, homogeneous intranuclear inclusions in nerve cell, one on either side of nucleolus

FIG 19 *Guinea pig 12 Cerebrum—anterior third—cortex* Focus of diffuse perivascular and leptomeningeal infiltration by round cells and mild associated microglial proliferation Hematoxylin-eosin stain

FIG 20 *Guinea pig 23 Spinal cord* Mild leptomeningitis on ventral surface of lumbar cord Infiltrating cells chiefly lymphocytes Hematoxylin-eosin $\times 460$

FIG 21 *Guinea pig 22 Spinal cord* Anterior horn of lumbar segment Mild perivascular infiltration by lymphocytes



DISTRIBUTION OF THE Rh FACTOR IN AMERICAN INDIANS

By KARL LANDSTEINER, M.D., ALEXANDER S. WIENER, M.D. AND G. ALBIN MATSON, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research and the Office of the Chief Medical Examiner of the City of New York, New York, and the Station Hospital, Fort Lewis, Washington)

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Among the numerous investigations on the racial distribution of the human individual blood properties A, B, and M, N, the most striking deviations from other races were found in American Indians, Eskimos, Australian aborigines, and Ainus¹. Since material from American Indians was available to us, it was considered of interest to study this with regard to the incidence of the Rh factor.

Materials and Methods

The clotted blood samples obtained by venepuncture at Fort Lewis, Washington, were shipped at once by air mail to New York City, where they arrived in good condition. These specimens came from Indian patients at the Tacoma Hospital and Indian soldiers stationed at Fort Lewis.

The Indians from which the samples were obtained fall into ten different linguistic groups with the exception of a few individuals. (This classification is based on the ethnological charts of linguistic stocks in Wissler's "The American Indian," 1938 edition, supplemented by information obtained from Dr. J. H. Hendry, Superintendent of the Indian Hospital, and Mr. O. C. Upchurch, Indian Agent at Tulalip, Washington.) The principal tribes of the individuals tested belonged to the following stocks: Shahaptin-Salishan, Klamath-Athabaskan, Siouan-Tlingit or Kolutshan, Haida, Shoshonean-Eskimaean, Algonkin.

Each Indian tested was classified according to the degree of Indian blood. The ratings as to racial purity are based on the agency records; these, naturally, may not be entirely accurate.

When the blood samples were received, even suspensions were prepared by breaking up the clots in saline solution, removing coarse particles, and washing once with saline. The tests for Rh were made with guinea pig immune sera (11) and with a human anti-Rh serum. For the latter tests, equal parts of a thin blood suspension and of serum were incubated at 37°C. All specimens designated as derived from

¹ Illustrative results selected from the literature can be found in the papers by Snyder (1) (A, B in Indians), Matson and Schrader (2) (A, B in Indians), Landsteiner and Levine (3) (M, N in Indians), Birdsell and Boyd (4) (A, B, M, N in Australian aborigines), Fabricius-Hansen (5) (A, B, M, N in Eskimos), Grove (6) (A, B in Ainus and Filipinos), Nigg (7) (A, B in Polynesians and Hawaiians), Kubo (8) (M, N in Ainus), and for general bibliography see Boyd (9) and Wiener (10).

full blooded and three-quarter blooded Indians were tested with both reagents, the others with human serum alone. In addition, the blood groups, sub groups, and M-N types were determined.

Some of the specimens from full blooded and three-fourths blooded Indians were examined with a human serum² which in whites gives about 27 per cent negative reactions instead of 15 per cent (11, 12).

Findings

The results presented in Table I demonstrate that the distribution of the Rh factor among American Indians differs widely from that among white Americans in New York City. While as many as 15 per cent of bloods from white individuals lack the Rh factor, only one such blood was found among 120 full blooded Indians and, of course, it cannot be concluded with certainty that even this single case was not attributable to some untraceable crossing

TABLE I
Distribution of the Rh Agglutinin among American Indians As Compared with White Individuals

Population examined	Rh positive		Rh negative		Total No examined
	Number	Per cent	Number	Per cent	
Full blooded Indians	119	99.2	1	0.8	120
Whites (New York City)					
Landsteiner and Wiener (11)	379	84.6	69	15.4	448
Levine <i>et al.</i> (22)		86		14	1035

with whites, a possibility which may also apply to the single group B individual found (*cf.* Table II). Among 69 Indians designated as three-fourths blooded all gave positive reactions with the exception of one blood, which was negative with the guinea-pig antiserum and with one human serum, but gave weakly positive reactions with three other human anti-Rh sera.

In the tests for the type demonstrable with the special human anti-Rh serum mentioned above, 69 specimens from full blooded Indians were examined. Of these, 29, or about two-fifths, showed negative reactions with this serum which is more than twice the frequency found among Rh-positive bloods from white individuals.

In Table II are given the figures for the distributions of O-A-B, A₁-A₂, and M-N. These confirm previous findings, especially the high frequency of group O and the low incidence of type N. The incidence of group A is higher in our material than in most Indian tribes, but considerably lower than in two tribes studied by Matson and Schrader (2), and it should be noted that, as in a

² For this serum we are indebted to Dr. Philip Levine.

previous study by Levine, Matson, and Schrader (13), all the group A full blooded Indians belonged to subgroup A₁.

The frequencies of the various blood factors in Indians known not to be of pure stock are presented in Table III, together with the frequencies to be anticipated from the degree of mixture with whites

TABLE II

Distribution of the Agglutinogens A₁-A₂-B, and M N among American Indians As Compared with American Whites

Population		Distribution of groups and subgroups						Distribution of M N types		
		O	A ₁	A ₂	B	A ₁ B	A ₂ B	M	N	MN
Full blooded Indians (Present study)	Number	88	31	0	1	0	0	68	5	47
	Per cent	73.3	25.8	0	0.8	0	0	56.7	4.2	39.2
American whites (Cited after Wiener (10))	Per cent	44.6	25.6	12.5	13.6	3.1	1.2	29.2	21.3	49.6

TABLE III

Distribution of the Agglutinogens Rh, A₁-A₂-B, and M N among American Indians of Mixed Ancestry

(Compared with expected distribution calculated from average degree of crossing with whites)

Population		Distribution of Rh		Distribution of blood groups						Distribution of M-N		
		Positive	Negative	O	A ₁	A ₂	B	A ₁ B	A ₂ B	M	N	MN
American Indians of mixed ancestry (Present study)	Number	148	7	90	49	5	7	4	0	75	11	69
	Per cent.	95.5	4.5	58.1	31.6	3.2	4.5	2.6	0	48.4	7.1	44.5
Theoretical distribution for populations with 43 per cent Indian blood	Per cent	93.5	6.5	56.1	25.9	7.4	8.4	1.6	0.5	40.4	13.2	46.3
	Per cent	95.6	4.4	61.0	25.7	4.4	6.6	1.2	0.3	45.4	10.6	43.9

It may be recalled that the expected distribution resulting from inter breeding can easily be computed from the number of individuals of the parent populations and the distribution of the blood properties in each of them. This has been done for the blood groups by Bernstein (14), who showed that the distribution in the mixed population can be obtained graphically by a method analogous to the location of the center of gravity of two masses. In this way, the frequency of the *rh* gene for half blooded Indians would be the average

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THE PATHOGENESIS AND PATHOLOGY OF EXPERIMENTAL TYPE I PNEUMOCOCCIC PNEUMONIA IN THE MONKEY*

By CLAYTON G. LOOSLI, M.D.

(From the Department of Medicine and the Douglas Smith Foundation for
Medical Research of The University of Chicago, Chicago)

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PLATES 6 TO 10

The monkey has been employed in only a few instances for the investigation of experimental pneumococcic pneumonia. Among the reported studies, in which this animal was used, those of Blake and Cecil (1) and Schöbl and Sellards (2) give detailed observations concerning the pathology and pathogenesis of the disease. Stuppy, Falk, and Jacobson (3) were concerned principally with the production of Type I pneumococcus pneumonia in the *Macacus rhesus* and *Cebus capucinus* species. Francis and Terrell (4) studied the production and clinical course of Type III pneumococcus pneumonia in the Java monkey, *Macacus cynomolgus*. They used both the intratracheal and intrabronchial methods of inoculation. With the intratracheal method it was noted that the inoculum was more widely distributed in the lungs than when the intrabronchial procedure was employed. Similar lesions, as observed by x ray, eventually developed, however, following either technique of production.

Blake and Cecil used the intratracheal method of inoculation during their studies and concluded that the resulting pneumonic infection simulated in every way that which occurred spontaneously in man. Concerning the mechanism of lobar consolidation, they, further, concluded that the pneumococci penetrated directly through the epithelium of the main bronchus of the lobe near the hilum and spread rapidly throughout the lobe by way of the perivascular and peribronchial tissues and lymphatics into the alveolar walls where they then passed into the air spaces simultaneously with the outpouring of the exudate. More recently Robertson and co-workers (5-7), Gunn and Nungester (8), Wood (9) and the author (10), using the intrabronchial method of inoculation and different animal species, also noted the similarity between the experimental pneumococcic infections and those which occur spontaneously in man, but their findings did not support those of Blake and Cecil, concerning the manner by which the organisms spread within the lungs. Rather they confirmed the views of Loeschcke (11) who concluded, from his studies of the pathogenesis and pathology of the pneumococcic pneumonia in man, that the pneumonic process was primarily intraalveolar and intrabronchial, and that the pneumococci were carried in the edema fluid directly from alveolus to alveolus through the pores of Kohn (12) and from bronchiole to bronchiole as a result of repeated aspirations, aided by breathing, coughing, and gravity.

* Aided in part by the Jessie Horton Koesler Fellowship Fund of the Institute of Medicine of Chicago, Chicago

The intrabronchial method of inoculation, as perfected by Terrell, Robertson, and Coggeshall (13) for their study of pneumococcic pneumonia in the dog, enables one to control and determine accurately the initial site or sites of involvement, thus making it better suited than the intratracheal technique for the study of the development of lobar consolidation. An investigation of the pathogenesis and pathology of pneumococcus pneumonia in the monkey, during which the intrabronchial technique of production was used, has not been reported. It seemed advisable to carry out such a study because the earlier views of Blake and Cecil, concerning the pathogenesis of lobar consolidation in the monkey, have not been substantiated by more recent studies of this phase of pneumonia occurring in man (11, 14-16) and induced, by this method, in other animal species. To compare accurately the development of lobar consolidation in this series of monkeys with that recently observed in the dog, the same general plan of infecting and sacrificing the animals, and preparing the lung specimens for microscopic study was followed (10).

Materials and Methods

Fourteen apparently healthy *Macacus rhesus* monkeys were used. Their lungs were shown to be free from tuberculosis by x-ray. The intrabronchial technique of infecting the animals was, briefly, as follows. The animal was placed under morphine anesthesia, and its larynx cocaineized, after which pneumococci suspended in a starch-paste medium were injected into the lungs through a radio-opaque ureteral catheter (No. 8), introduced through the trachea, into one of the lower lobe bronchi, with the aid of a fluoroscope. Type IA₂ strain of pneumococcus was used as the infecting agent. This organism, when injected intraperitoneally, killed mice regularly in dilutions of 10^{-8} . The dosage varied from 0.01 cc. to 0.02 cc. of an 18 hour broth culture suspended in $\frac{1}{2}$ cc. of 5 per cent starch-broth mixture. Monkey 1-37, killed $1\frac{1}{2}$ hours after inoculation, received 0.4 cc. of pneumococci suspended in 2 cc. of starch in two sites in each lower lobe.

The clinical course of the disease was followed by daily temperature, pulse, and leucocyte counts. X-rays were taken at 24 hours on those animals allowed to survive longer than this time. Blood cultures were taken on eight of the animals.

The animals were sacrificed at $1\frac{1}{2}$, $2\frac{1}{2}$, $4\frac{1}{2}$, 9, $14\frac{1}{2}$, 22, 28, 36, 48, 60, 70, 72 hours, 4 and 7 days after inoculation. Each animal was killed with a lethal dose of pentobarbital sodium, after which its chest was quickly opened, the aorta clamped, and a ligature placed about the base of the heart to keep the blood in the pulmonary vessels. The pneumonic lungs were then filled with Zenker-formalin fixing solution (10 per cent formalin in Zenker's fluid without acetic acid) through the trachea and allowed to fix for from 6 to 10 hours. After this time specimens were cut from the involved lobes, washed, dehydrated, embedded in celloidin, sectioned (8 to 10 micra), and placed serially on slides, after which they were stained by Maximow's hematoxylin-eosin-azure II method (17).

Clinical Observations

The animals remained under the effect of the morphine for about 14 hours. The nine animals killed after 22 hours appeared ill. They failed to eat and were less active. Their respiratory rates were increased and some exhibited pain in the chest when handled. At 24 hours all nine animals had a leucocytosis and elevated temperatures. Two of the animals killed at 2½ and 4 days after infection were moribund and had subnormal temperatures and low leucocyte counts. All the other animals had a leucocytosis at the time of sacrifice, except the one killed the 7th day after inoculation and 3 days after recovery.

Blood cultures were taken on the animals killed at 14, 22, 36, 48, 60, 72, 96 hours and 7 days after infection, and all were positive for Type I pneumococci. Monkey 76 killed after 2½ days of infection had over a thousand colonies at 24 hours and at the time of sacrifice 370 colonies per cc. of blood. Monkey 81 had 16 colonies per cc. of blood at 24 hours and thousands at the time of sacrifice 4 days after inoculation. Monkey 82 sacrificed at 7 days had 188 colonies per cc. of blood at 24 hours, and at 5 days its blood was sterile. The remaining animals on which cultures were taken had thousands of colonies per cc. of blood at the time of sacrifice.

Pathogenesis of the Lesions

Macroscopic Observations—As the lungs of all the animals were fixed intratracheally, immediately after death, only a hurried inspection of the pleural surfaces of the pneumonic lesions was made. The maximum consolidation occurred within 22 hours after inoculation. Monkey 132 killed at 48 hours had approximately ¼ of the inoculated lobe consolidated. All the other animals killed after 22 hours had ¾ or more of the inoculated lobes consolidated. Spread of the infection to other lobes occurred only in monkey 139, killed at 14 hours. In this animal portions of the left upper and left lower lobes as well as the inoculated right lower lobe were consolidated.

The inoculated lobes of the six animals killed at increasing intervals within 22 hours after infection showed a regular increase in the extent of consolidation which progressed from the periphery of the lungs at the site of inoculation toward the hilum. The pneumonic areas, enlarging in a contiguous manner, were deep red, slightly retracted, irregular in outline, and edematous at their spreading margins. In all the animals there was a sharp line of demarcation between the consolidated and unconsolidated portions of the lobes. The consolidated lobes were always smaller than the corresponding opposite uninvolved lobes. Fibrin was present on the pleural surfaces in some of the animals. The consolidated lobes of the animals killed within 28 and 72 hours after inoculation had essentially the same grayish red appearance. Monkey 82 killed at 7 days after infection had recovered and the inoculated lobe was air-containing and showed only slight discoloration.

Microscopic Observations—The inflammatory reaction had already begun 1½ hours after inoculation (Fig. 1). It had a bronchial or focal distribution. The initial exudate consisted of edema fluid in which were a few red blood cells and blood leucocytes and pneumococci. There was no detachment of the septal cells but the capillaries were greatly congested with leucocytes (Fig. 1). At 2½ hours the focal areas

of inflammation were larger and showed large numbers of leucocytes leaving the septal capillaries and entering the air spaces (Figs 2 and 3). At 4½ hours the alveoli and bronchi were more or less filled with the serous and cellular exudate, although the extent of consolidation was small (Fig 4). The margins of the consolidated areas showed only an edematous exudate and a beginning migration of leucocytes from the blood as was present in the 1½ hour lesion. The borders of the increasingly larger areas of consolidation in the lungs of the animals killed at 9, 14½, and 22 hours had this same appearance (Figs 9 and 10) with the older portions showing increasing numbers of cells in the exudate (Figs 8, 11, and 12). Twenty-two hours after infection the alveoli and bronchi were more or less uniformly filled with exudate (Fig 12).

The serum and blood leucocytes entered the air spaces principally through the small blood vessels, and capillaries in the alveolar walls (Fig 3). There was some perivascular and peribronchial edema and varying degrees of interstitial cellular infiltration in different parts of the lung lesions of the six animals killed during the first 24 hours of the disease when maximum consolidation took place. The interstitial reaction, however, bore no relation to the extent or intensity of consolidation. Areas showing no interstitial reaction (Fig 12) were as intensely consolidated as other regions which also showed peribronchial and perivascular edema and cellular infiltration. Interstitial and lymphatic involvement were more pronounced in the 1½ to 4 day lesions, yet the reaction within the air spaces during this time was one of gradual recovery (Figs 15 to 19).

Distribution of Pneumococci.—In the consolidated lobes of the five animals killed within the first 15 hours after inoculation, pneumococci were seen only in the alveolar and bronchial exudates and in large numbers after 4 hours (Figs 6 and 7). The greatest number of organisms were in the alveolar and bronchial spaces at the spreading border of the lesions where the exudate consisted principally of edema fluid (Figs 6, 9, and 10). In the older consolidated portions of the lesions organisms were seen in varying numbers both free and in leucocytes. No organisms were seen in the substance of the alveolar walls in any of the developing lesions, but beyond their margins many were lying in a thin layer of edema fluid on the alveolar surfaces and in the pores of Kohn of apparently normal alveoli (Fig 6). Likewise, none were seen at points in the septa where the leucocytes were entering the spaces (Fig 3). Pneumococci were not seen invading the bronchial epithelial cells, although in the early lesions large numbers were seen in the exudate lying on their free surfaces.

An occasional pneumococcus was seen in the edematous interstitial tissue of some bronchial and blood vessel walls in the lesions of the animals killed at 28, 36, 48, 60, 72, and 96 hours after infection. Pneumococci were not seen in the small or large perivascular, peribronchial, or pleural lymphatics in the lesions of any of these animals and were demonstrable only in the blood vessels and septal capillaries of two animals which were moribund and had marked bacteremias at the time of sacrifice, 48 and 96 hours after inoculation. Only an occasional pneumococcus was seen in the blood of the animal killed at 48 hours, while large numbers were present both free and in polymorphonuclear and monocytic leucocytes within the vessels and capillaries of the one killed at 4 days after infection (Fig 19). Small thrombi containing large numbers of organisms were present in the lung capillaries and larger pulmonary vessels of this animal.

In spite of the increased and persistent interstitial inflammation of the pneumonic lungs (Figs. 17 and 18) and bacteremias in the animals killed after the first 24 hours of the disease, pneumococci diminished in numbers from the bronchial and alveolar exudate. They were found only in small numbers in the exudates of the animals killed at 28, 36, 48, and 60 hours, and were not seen in the exudates of those killed at 3, 4 and 7 days after infection. At the same time the organisms disappeared from the exudate, the cellular elements changed gradually from polymorphonuclear leucocytes to large markedly phagocytic mononuclear cells.

Histogenesis of Cells in the Exudate

With the exception of a few old, free, carbon filled macrophages, which are normally present in the alveoli, the blood leucocytes were the first cells to appear in large numbers in the exudate. Within 1½ hours after inoculation polymorphonuclear leucocytes had collected in large numbers in the capillaries and small blood vessels in the alveolar walls, and a few were already in the air spaces (Fig. 1). The migration of leucocytes from the capillaries and blood vessels followed rapidly the outpouring of edema fluid into the alveolar spaces and continued until the greater portion of the inoculated lobes were consolidated. The leucocytes entered the alveoli principally through the septal capillaries and small blood vessels (Fig. 3). Leucocytes in all stages of passing from the alveolar capillaries could be seen throughout the lesions and especially in areas near the margin of the spreading borders in the animals killed during the first 24 hours. As they entered the air spaces, the leucocytes, both granular and non-granular varieties formed asteroid configurations along the septa (Fig. 3). In some areas a few passed directly through the larger pulmonary vessels and vessels in the bronchial walls into the edematous interstitial tissue, but leucocytes were present only in relatively small numbers in the interstitial tissue in the lesions of the animals killed within 24 hours after inoculation (Figs. 1 to 12). The polymorphonuclear leucocyte was the predominant cell type in the exudate during the first 36 hours after infection. During this time they phagocytized varying numbers of pneumococci and began to degenerate so that by 72 hours the majority were degenerating and being ingested by the mononuclear macrophages. None were present in the alveolar spaces of the clearing 7 day lesion.

The non-granular leucocytes (lymphocytes and monocytes) entered the exudate along with the polymorphonuclear leucocytes throughout the early stages of the disease. At first they were inconspicuous in the exudate because of the predominance of polymorphonuclear leucocytes. Only a few were in the exudate in the 2½ hour lesion (Fig. 3) but they became increasingly more numerous in the 4½, 9, and 14½ hour lesions (Figs. 7, 8, and 11). After entering the exudate the hematogenous mononuclear exudate cells did not degenerate, but soon began to hypertrophy and transform themselves into actively phagocytic cells. The morphological transformation of the lymphocytes into monocytes and monocytes into macrophages was clearly seen by examining the exudates in consecutively older lesions within the first 36 hours of the disease. In the 9 hour lesion (Fig. 8) the mononuclear exudate cells were larger and more numerous than in the 4½ hour lesion (Fig. 7) but the majority of these cells at this time resembled the lymphocytes still within the blood vessels

In the 14½ hour lesion (Fig 11), they were definitely larger than those seen in the 9 hour lesion (Fig 8). Their nuclei were less compact and assumed many shapes. They had accumulated more cytoplasm which was less basophilic than that of the cells in the 9 hour lesion. Many of these cells resembled monocytes of the blood, but the great majority of the cells still within the vessels were of the lymphocytic variety. The larger of the "polyblastic" mononuclear cells in the 14½ hour lesions had begun to phagocytize red blood cells and an occasional pneumococcus. In the 22 hour lesion, near the site of inoculation (Fig 13), the mononuclear exudate cells were still larger and more phagocytic than those seen at 14½ hours. In the exudate at the margin of the 22 hour lesion (Fig 14) which was more recently involved, they were smaller and resembled those in the 9 and 14½ hour lesions.

By 28 hours the majority of the lymphocytes and monocytes had assumed the morphology and function of macrophages which were still larger and actively phagocytizing large numbers of degenerating granular leucocytes, red blood cells, and pneumococci in the 36 hour lesion (Fig 15). After this time only an occasional small hematogenous mononuclear cell was seen in the exudate, which consisted principally of typical large mononuclear cells with ingested constituents, in various degrees of digestion in their cytoplasm (Fig 16). During the first 36 hours of the disease when the transformation of the non-granular leucocytes into larger phagocytic cells took place, the local tissue cells showed only swelling and no proliferation. There was no evidence in any of the lesions that the hematogenous mononuclear exudate cells degenerated after they entered the exudate.

The septal cells (alveolar epithelium) did not become detached or show any significant change as a result of the marked exudation of serum and leucocytes from the blood during the first 24 hours of the disease (Figs 3 to 13). The enlargement of the septal cells was gradual and associated with swelling of other cellular constituents (capillary endothelium and fibroblasts) in the alveolar walls. By 36 hours septal cells were conspicuous on the walls where they had accumulated large amounts of foamy cytoplasm, but no mitotic figures indicating proliferation were seen in them (Fig 13). Mitotic figures were rarely seen in these cells, and then only in the 2½, 3, and 4 day lesions. At this time binucleated cells appeared on the alveolar walls. These binucleated cells were still numerous on the septa in the 4 and 7 day lesions which were undergoing resolution and showing diminishing numbers of macrophages in the exudate (Figs 18 to 20). No phagocytosis of pneumococci, red blood cells, and polymorphonuclear leucocytes by the attached single or binucleated septal cells was noted in any stage of the disease. Single or binucleated mononuclear cells, which did not contain ingested material in their cytoplasm and which resembled the attached cells, were seldom seen free in the spaces. The septal cells remained enlarged and numerous on the walls in the 4 and 7 day lesions which were undergoing resolution (Figs 19 and 20).

The bronchial epithelium showed no injury, proliferation, or phagocytic activity in any of the lesions, although it became infiltrated with polymorphonuclear leucocytes in the early stages of the disease. The endothelial cells lining the pulmonary vessels, capillaries, and lymphatics, showed no proliferative activity at any stage, or phagocytosis of organisms in the 2 and 4 day lesions in which intravascular pneumococci could be seen. There was no evidence of proliferation of the fixed tissue histiocytes

in the interstitial tissue of the bronchi, pleura, and blood vessels with their subsequent migration into the alveolar spaces to contribute to the source of the mononuclear macrophages in the exudate. Likewise, as far as could be determined, the old pigment filled macrophages in the interstitial tissue did not migrate into the exudate in response to the acute infection. On the other hand, the resolving lesions showed an increased number of macrophages in the interstitial tissue. These were morphologically identical with the diminishing macrophages still in the alveolar spaces. A few smaller basophilic mononuclear cells accumulated in the interstitial tissue in the older lesions. These resembled lymphocytes, monocytes, and plasma cells.

DISCUSSION

The clinical manifestations of pneumonia in this series of animals were similar to those observed by Blake and Cecil (18), Schöbl and Sellards (2), and Francis and Terrell (4) in larger series of monkeys, and by Terrell, Robertson, and Coggeshall (13) in experimental pneumonia in the dog. Although most of the animals were killed within 4 days after inoculation, the early severe bacteremias in all but one, on which cultures were taken, indicated that, if allowed to survive longer, the majority would have eventually succumbed to the infection. At the time of sacrifice the animals varied greatly in severity of illness. The histological findings, however, could be more closely correlated with the age of lesions, than with their gross appearance or the clinical condition of the animals at the time of death.

The evolution of the inflammatory process in the lungs was rapid, progressive, and similar to that seen by Robertson, Coggeshall, and Terrell (5), and the author (10) during their studies of pneumonia in the dog. Lobar consolidation took place within 24 hours after inoculation. The consolidated lobes of animals killed within the first 72 hours had essentially the same gross appearance, but during this time, the exudate changed gradually from one composed of edema fluid, polymorphonuclear leucocytes, lymphocytes, monocytes, red blood cells, and many pneumococci to one of large mononuclear macrophages. By the 4th day resolution was well advanced, and practically complete by the 7th day. The cellular changes in and the disappearance of pneumococci from the exudate took place in spite of early persistent bacteremias and increasing amounts of interstitial and lymphatic involvement. Similar changes were reported by Robertson and Loosli (19) in the pneumonic exudate in dogs showing severe infections and dying on or after the 4th day of illness. Francis and Terrell also noted by x ray in monkeys that lobes which became consolidated by 24 hours after inoculation showed varying degrees of clearing on the 3rd or 4th day of the disease, although during this time some of the animals had developed bacteremias and had a spread of their infections to other lobes.

The histological changes, the kinds of cells, and the sequence with which they appeared in the exudate were similar to those seen in the induced pneu-

monic lesions in dogs (10) The polymorphonuclear leucocytes appeared early and in larger numbers, but they began to degenerate and be taken up by the mononuclear macrophages after 24 hours The mononuclear exudate cells presented a great variety of sizes, shapes, and degrees of phagocytic activity depending on the age of the consolidations By observing them in consecutively older lesions, it could be seen that the "polyblastic" mononuclear cells represented transitional stages in the development of the small hematogenous mononuclear exudate cells (lymphocytes) which appeared early in the exudate, into larger mononuclear phagocytes The transformation of the lymphocytes into monocytes and monocytes into typical macrophages was more or less completed during the first 36 hours of the disease By examining only lesions of animals killed after this time, one could readily get the impression that the large mononuclear phagocytic cells arose only locally from the swollen septal cells, as is generally considered to occur both in the case of pneumonia in man and experimental pneumonia in animals (10)

It was impossible, however, to determine to what extent the septal cells contributed to the source of the free macrophages, for large numbers of the latter cells, which were derived from the hematogenous mononuclear cells, were present in the exudate before the local tissue and septal cells began to show a reaction to the invading organisms By studying the septal cells in consecutively older lesions from the beginning of consolidation until resolution was complete, it appeared that their reaction was one principally of swelling with occasional division to form binucleated attached cells Similar observations were noted during the study of the septal cell reactions in pneumonic lesions in dogs A more extended consideration of the histogenesis of cells in pneumococcic pneumonia has been given in this report (10)

The observations concerning the development of lobar consolidation in this series of monkeys were, likewise, similar to those noted during the study of the histogenesis of cells in experimental pneumonia in the dog (10) In the early lesions the pneumococci were distributed principally within the air spaces, and were most numerous in the edematous exudate, which was abundant, at the spreading borders of the consolidated areas The progressive and contiguous enlargement of the areas of inflammation occurred as a result of the spread of the infected edema fluid, which preceded the entrance of leucocytes into the air spaces, from alveolus to alveolus directly through the pores of Kohn (12) and from bronchiole to bronchiole by repeated aspirations These findings are in agreement with those of Loeschcke (11), Heinrichs (14), Robertson and Uhley (15), and Loosh (16) concerning the pathogenesis of lobar consolidation in man, and also with the recent observations made by Robertson (6), Robertson and Hamburger (7), during similar studies in the dog, and by Gunn and Nungester (8), and Wood (9) in the rat These studies as well as the present one show that pneumococcic pneumonia is primarily

an intraalveolar infection. They further show that the injury to the alveolar tissue and capillaries, which is followed by exudation of the blood constituents into the air spaces, can best be attributed to the soluble toxic products liberated by the pneumococci growing in the exudate rather than by direct invasion of the septal tissue by the organisms themselves, as Blake and Cecil, Schöbl and Sellards, Stillman and Branch (20), and Rake (21) concluded.

The outpouring of edema fluid appears to be typical of the body's reaction to the pneumococcus regardless of the site of inoculation as has been shown by Welch (22) and Goodner (23) in their studies of dermal pneumonia in the rabbit. That substances liberated by the pneumococcus can produce an edematous reaction when injected into the skin of man and the lungs of animals has been shown recently by Dick and Boor (24), and Suthiff and Friedemann (25). Whatever the nature of this edema producing substance or substances might be, their injurious action is mild, for consolidation is seldom followed by necrosis of the lung tissue. The abundant fluid exudate in pneumococcal inflammations most likely does not represent a manifestation of allergy, as Loeschcke, Lauche (26), Fried (27), Heinrichs, and Lindau (28) maintain. Similar inflammatory reactions can be elicited by the pneumococcus in non-sensitized and sensitized, and in immune and non immune animals. There is no evidence that it represents an allergic reaction in the case of pneumococcal pneumonia in man. Likewise, there was no evidence in this study to support the concept of a neurogenic factor in the production of the edematous exudate in pneumonic lesions as proposed by Reinhardt (29).

Atelectasis may serve to hold the organisms in the lungs until the inflammatory process is initiated, but it probably does not play an important part in the spread of the infection within the air spaces as Coryllos and Birnbaum concluded (30). Rather, it would seem that atelectasis should prevent or hinder the spread of organisms within the air spaces.

The rapidity with which lobar consolidation occurred in this series of animals, and the distribution of the pneumococci in the consecutively older lesions would indicate that the data upon which Blake and Cecil based their concept of pathogenesis of lobar consolidation in the monkey were incomplete. They made no systematic study by x ray of the evolution of the lesions as did Francis and Terrell, nor did they sacrifice animals at increasing intervals of time after inoculation, so that the age of the lesions could be accurately determined. Although Blake and Cecil examined the lungs of two animals sacrificed at 3½ and 12 hours after inoculation, their observations concerning the manner of intrapulmonary spread of the organisms, for the most part, were made from lungs of animals dying with pneumococcal septicemias from 3 to 5 days after inoculation.

Schöbl and Sellards, and Permar (31) also considered the interstitial tissue and lymphatics to be the chief pathways for the spread of the organisms within

the lungs in the early stages of the disease when consolidation took place Schobl and Sellards, however, examined the lungs of animals dying only after the 1st day of the disease. They observed no organisms but only a cellular infiltration in the interstitial tissue and congested lymphatics. Permar, using rabbits, observed organisms in the interstitial tissue and lymphatics of the lungs of animals killed only 10 hours or longer after infection. In the present study as well as that made in dogs (10), organisms were never seen in the developing lesions in the substance of the alveolar walls, in the interstitial tissue of the bronchial tree, or penetrating the bronchial epithelium as Blake and Cecil describe. Large numbers, however, were present in the bronchial and alveolar exudate of lesions comparable to their early ones. The manner of fixing the lungs for microscopic examination in this series of animals, however, aided materially in determining whether the organisms were in the tissues or air spaces. The results of this study of the distribution of the pneumococci in such prepared lungs suggest that the great majority of organisms which Blake and Cecil considered to be in the walls of the terminal bronchioles, alveolar ducts, and alveoli in their early lesion were in reality lying on the surfaces of these partially collapsed structures. In early pneumonic lesions in man pneumococci also have been infrequently noted in the interstitial tissue while large numbers have been observed in the fluid exudate in the air spaces (11, 15, 16, 33, 34). That pneumococcic inflammation of the lungs should be considered an infection of the alveolar and bronchial surfaces, as Wadsworth (35) long ago pointed out, is substantiated in this study.

Varying degrees of interstitial and lymphatic involvement were noted in this study, but it could not be correlated with the extent or degree of consolidation. Interstitial involvement was more pronounced in the lungs of the animals killed after consolidation had taken place, yet in these lesions organisms gradually disappeared from the exudate, as it changed from one of polymorphonuclear leucocytes to one of mononuclear macrophages. The exudate in the animal killed at 4 days was undergoing resolution in spite of a marked interstitial reaction and a bacteremia so pronounced that organisms could be seen in large numbers in the septal capillaries and larger pulmonary vessels. Thus the presence of organisms and exudate cells in the interstitial tissue, and organisms in the alveolar walls of animals dying of severe septicemias after 3 or 4 days of illness does not necessarily indicate an inflammatory process which will lead to consolidation of the lungs, as Blake and Cecil concluded. Certainly organisms do enter the interstitial tissue and lymphatics in varying numbers, but they do so in all probability secondarily from the alveolar spaces. Once in these channels they most likely do not reenter the alveoli, but become important as the source of infection producing complications, such as pericarditis, empyema, and bacteremia, rather than having a

part in the pathogenesis of lobar consolidation. Recent studies concerning the lung blood barrier in experimental pneumonia in the dog further substantiate this view (36)

Blake and Cecil's observation, that the pneumococci entered the interstitial tissue first through the bronchial epithelial lining, was not confirmed by Schöbl and Sellards, Permar, and Gaskel (32), who used the same or similar techniques of inoculation. They concluded that the fluid inoculum was aspirated directly into the finer air spaces. They further pointed out that, following the intratracheal injection of organisms, consolidation began at or near the hila of the lobes because the fluid inoculum, with the animals on their backs, entered directly into the most dependent short bronchial alveolar units in the hilar regions and that the position of the animal determined which lobes the inoculum entered. Recently Robertson and Hamburger (7) have shown in the dog that the pneumonic exudate must be fluid enough to flow or be aspirated into the terminal air passages before secondary pulmonary lesions develop. Viscid exudate, containing large numbers of organisms, placed in the entrance of primary bronchi with the animals in a position favorable for flow of the material into the lobe did not result in pulmonary consolidation. There was, however, ample opportunity for organisms in the exudate to penetrate the bronchial epithelium and produce interstitial inflammation and consolidation. That this did not occur is further evidence against the validity of the concept of pathogenesis of lobar pneumonia as proposed by Blake and Cecil.

SUMMARY

The pathogenesis of lobar consolidation and microscopic pathology of induced Type I pneumococcal pneumonia in a series of fourteen monkeys, killed at close intervals of time after infection, have been studied. The inflammatory process which resulted in consolidation was primarily intraalveolar and intrabronchial. The pneumococci spread within the air spaces as a result of the dissemination of the infected edema fluid directly from alveolus to alveolus through the pores of Kohn and from bronchiole to bronchiole as a result of repeated aspiration during breathing. The pneumonic process within the air spaces developed and progressed independently of the reaction in the interstitial tissues. The organisms spread to the interstitial tissue secondarily from the alveolar spaces. Once in the interstitial tissues they appeared to be the important source of infection producing bacteremia but not important in the mechanism by which consolidation was produced.

The exudate cells came chiefly from the blood. The large mononuclear cells which replaced the polymorphonuclear leucocytes were derived principally from the hypertrophy and transformation of lymphocytes and monocytes into

macrophages after they entered the exudate in the early stages of the disease. The part the local septal cells played as the source of the macrophages could not be accurately determined. The reaction of the septal cells appeared to be chiefly one of swelling without detachment and occasional proliferation to form binucleated attached cells. To follow the transformation of the hematogenous mononuclear cells into macrophages in the exudate, the inflammatory reaction must be examined at frequent intervals during the first 36 hours of the disease.

The similarity of the pathogenesis of lobar consolidation in human pneumonia to that observed in the experimentally induced disease in monkeys and dogs was discussed.

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EXPLANATION OF PLATES

Photomicrographs were made from sections stained with Maximow's hematoxylin-eosin-azure II technique

PLATE 6

FIG 1 Monkey 1-37M, killed $1\frac{1}{2}$ hours after left lower lobe inoculation Area at site of inoculation shows a beginning inflammatory reaction Some septal capillaries are markedly congested with leucocytes, and some leucocytes are already in the alveoli The granular precipitate is starch granules and serous exudate There is no involvement of interstitial tissue of blood vessel (*v*) or of the lymphatics (*l*) $\times 112$

FIG 2 Monkey 1-31M, killed $2\frac{1}{2}$ hours after right lower lobe inoculation Area near site of inoculation shows an intense inflammatory reaction The exudate consists of edema fluid, red blood cells, and leucocytes The vessel wall (*v*) shows no reaction although leucocytes have collected at the periphery of its lumen $\times 112$

FIG 3 Monkey 1-31M Area shows large numbers of leucocytes in the process of entering the exudate at points along the septa $\times 340$

FIG 4 Monkey 1-32M, killed $4\frac{1}{2}$ hours after right lower lobe inoculation Area near site of inoculation shows continued exudation of leucocytes into the air spaces at points along the septa The bronchial (*b*) and blood vessel (*v*) walls show only slight involvement. The lymphatic (*l*) is congested $\times 112$

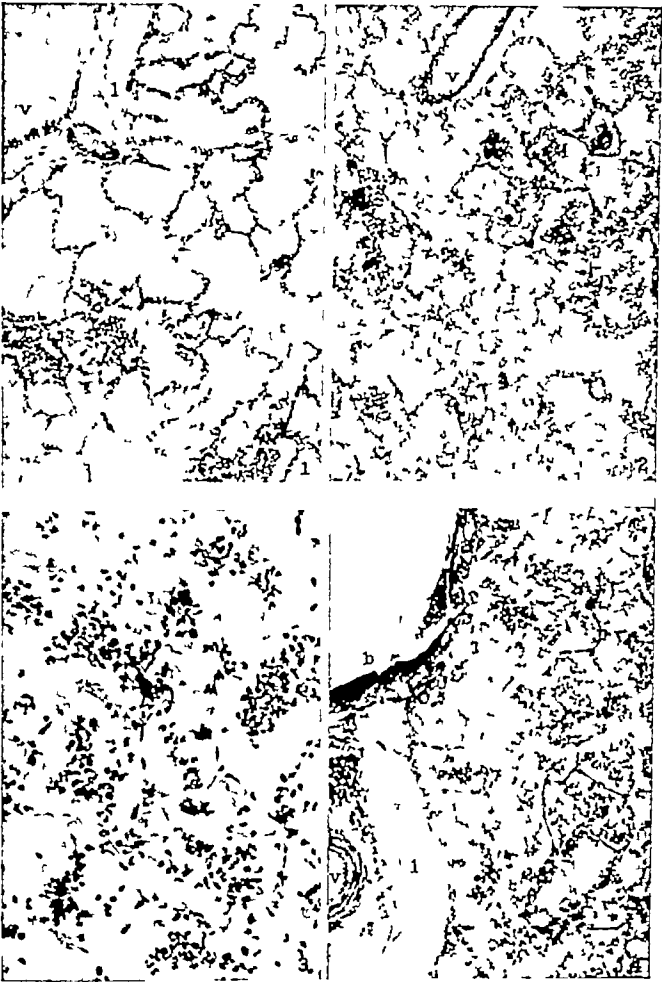


PLATE 7

FIG 5 Monkey 1-32M Area beyond the marginal zone of edema-filled alveoli. Septal walls appear normal. Small amount of serous precipitate in some alveoli. $\times 112$

FIG 6 Monkey 1-32M Area τ of Fig 5 shows many pneumococci lying in the thin layer of edema fluid on the surface of an alveolus. No organisms are seen in the substance of the walls. $\times 755$

FIG 7 Monkey 1-32M Area near the site of inoculation. Large numbers of pneumococci are present in the exudate. None are seen in the substance of the septa. A group of leucocytes (*a*) are entering the exudate. A few small mononuclear leucocytes are already in the exudate. One old pigment-filled macrophage (*m*) is present in the exudate. $\times 525$

FIG 8 Monkey 94M, killed 9 hours after right lower lobe inoculation. Area near site of inoculation shows the character of exudate. Pneumococci are seen but are fewer in number than at $4\frac{1}{2}$ hours (Fig 7). Cellular exudate consists principally of polymorphonuclear leucocytes, some containing ingested pneumococci. Numerous small hematogenous mononuclear cells are also present. Some have taken up an occasional pneumococcus. The alveolar walls appear normal. The septal cells (*s*) show no reaction. $\times 600$

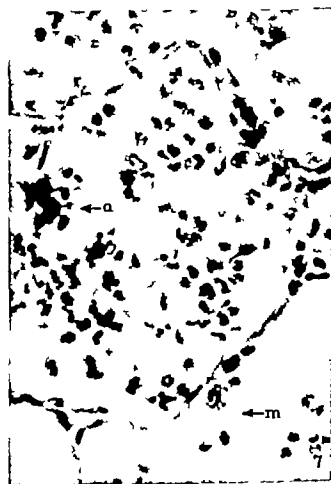
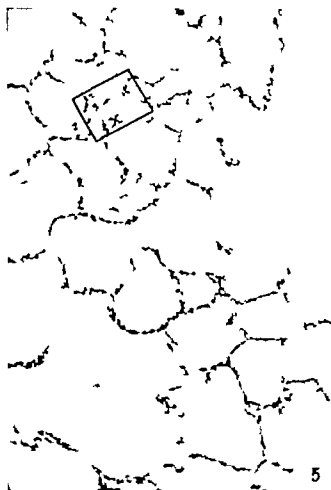


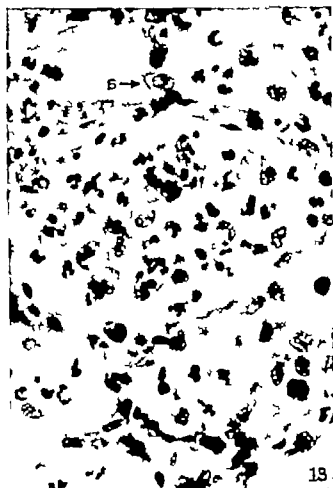
PLATE 9

FIG 13 Monkey 1-44M Area near site of inoculation shows the character of the exudate. A few polymorphonuclear leucocytes have begun to degenerate. The majority of the mononuclear cells are larger than those seen at 14 hours (Fig 11) and are actively phagocytic. A few smaller non-phagocytic hematogenous mononuclear cells of varying sizes, representing transitional stages in their development into macrophages, are seen. The septal cells (*s*) are beginning to enlarge. $\times 600$

FIG 14 Monkey 1-44M Area of more recent consolidation near the margin of the lesion shows numerous small mononuclear exudate cells similar to those within the blood vessels and in the exudate of earlier lesions (Figs 8 and 11). $\times 600$

FIG 15 Monkey 1-06M, killed 36 hours after right lower lobe inoculation. Area near the site of inoculation shows mononuclear exudate cells all of the large variety, which show marked phagocytic activity. Free pneumococci are not seen in the exudate or in the substance of the alveolar walls. The septal cells (*s*) are larger than those seen in the 22 hour lesion (Fig 13). $\times 600$

FIG 16 Monkey 76M, killed 2½ days after left lower lobe inoculation. The exudate cells consist principally of mononuclear macrophages which contain fragments of ingested polymorphonuclear leucocytes, red blood cells, etc. The alveolar walls are greatly swollen, and the septal cells (*s*) are conspicuous. No pneumococci are seen in the substance of the alveolar walls. $\times 600$



FUNCTION OF COMPONENTS OF COMPLEMENT IN IMMUNE HEMOLYSIS*

By L. PILLEMER, PH.D., S. SEIFTER, FEY CHU M.D., AND E. E. ECKER, PH.D.

(From the Institute of Pathology Western Reserve University and the University Hospitals, Cleveland)

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Although the activity of complement is exhibited in bactericidal, bacteriolytic, and hemolytic phenomena, the hemolysis of sensitized red blood cells is the most clearly defined property. Nevertheless, the rôle of the various components of complement in immune hemolysis has not been determined under the most favorable conditions.

Liefmann and Cohn (1) assert that the disappearance of complement in immune hemolysis is due to the effect of hemolysis rather than to an initial fixation of complement. On the contrary Eagle (2, 3) and Ponder (4) state that complement must first be bound by sensitized erythrocytes before hemolysis occurs. These authors made no attempt to identify the components operating in this fixation. Many investigators (5-8) have claimed that the mid-piece is fixed by sensitized red blood cells. Brin (9) states, however, that the end-piece alone is bound by immune aggregates. Still others hold that both end-piece and mid piece combine (10-12). It has also been asserted that the fourth component is involved in the fixation of complement prior to hemolysis (13-15). Furthermore, Nathan (16) concludes that the third component of complement is not fixed by sensitized sheep red blood cells.

Pillemer, Seifter and Ecker (17) have shown that C'4, C'2 and varying amounts of C'1 are always fixed by antigen rabbit antibody aggregates. In agreement with Nathan, these authors showed that C'3 is not fixed by sensitized red blood cells.

Fixation of the components of complement in the work last mentioned was determined by measuring the residual component activity in fresh guinea pig serum after fixation. This, however, did not disclose whether the adsorbed components were functionally active, since under the experimental conditions employed no visible changes occurred which could be attributed to the action of combining complement components. However, if the immune aggregate employed in fixation is a substrate which upon resuspension and appropriate treatment shows a visible manifestation, such as hemolysis, the

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¹ The symbols C'1, C'2, C'3 and C'4 refer to mid-piece, end piece, third component, and fourth component respectively in the older terminology (18). In the present paper the term "mid piece" is used to indicate the CO₂-insoluble fraction of complement (which may often contain C'1, C'3, and C'4); the term "end piece" is applied to the CO₂-soluble fraction which may contain C'2, C'3 and C'4.

nature and rôle of the fixed complement components can then be determined. The present paper presents the results of an investigation in which sensitized sheep red blood cells were used as an immune system to determine the rôle of the complement components in immune hemolysis.

Materials

Complement—Immediately after withdrawal, the blood of normal guinea pigs which had been maintained on a high vitamin C diet was placed in the ice box ($3-4^{\circ}\text{C}$) for about 1 hour. The serum was then separated by centrifugation and used promptly in the experiments. In the experiments reported here the serum was never allowed to stand more than 1 hour before use.

A unit of complement was considered to be the smallest amount of a 10 per cent dilution of fresh serum which caused 100 per cent hemolysis of 1 cc. of a 2.5 per cent suspension of sheep red blood corpuscles containing 5 units of anti-sheep cell rabbit serum per cc.

Sheep Red Blood Cells—Defibrinated sheep blood was strained through cotton gauze and washed four times with 10 volumes of physiological saline. After the final washing the cells were left in a thick suspension, each cubic centimeter of which contained approximately 10 billion cells as determined by cell count. Care was taken that the bloods employed were obtained daily from the same sheep so as to avoid, as far as possible, errors due to variations in the resistance of red blood cells to the action of complement.

1 cc. of a 2.5 per cent dilution of the thick suspension, or 250,000,000 erythrocytes, was taken as a unit of sheep red blood cells.

Anti-Sheep Cell Rabbit Serum—The anti-sheep red blood cell rabbit serum contained 10,000 units per cc. It was not anticomplementary in twice the quantities employed. A unit of anti-sheep cell rabbit serum was considered to be the smallest amount of antiserum necessary for the complete hemolysis of 250,000,000 red blood cells in the presence of 1 unit of complement. Nevertheless, 5 units of antiserum was always added to each unit of cells in order to avoid errors due to the possible presence of a natural hemolysin in guinea pig serum.

Methods

Specifically Inactivated Complements—Methods for the preparation and assay of these have been previously described (17, 26).

In estimating the degree of fixation of complement or its components by sensitized red blood cells, most workers have allowed complement to react with red cell aggregates for a few seconds, since longer periods at the temperatures employed by them (either room temperature or 37°C) would have resulted in hemolysis, and consequent overshadowing of exact fixation data. The present authors (17) have shown that 60 minutes treatment at 1°C results in almost complete fixation of the combining components of complement, differing only slightly from fixation at room temperature. In the experiments reported here fixation was carried out at 1°C for 1 hour, thus achieving fixation without hemolysis.

General Method for the Determination of the Rôle of the Components of Complement

in Immune Hemolysis—The reactants (complement, red cells, and antiserum) were combined in amounts previously estimated to contain 40 units each of complement and red cell substrate and 200 units of antiserum.

Therefore, to 1 cc. of packed red blood cells (containing 10 billion cells per cc.) 40 units of undiluted complement² were added with mixing, and the mixture was chilled to 1°C. 1 cc. of diluted antiserum containing 200 units, previously chilled to 1°C, was then added and the contents thoroughly mixed. The mixture was allowed to stand for 1 hour at 1°C. At the end of this time it was centrifuged in an angle centrifuge at 2750 R.P.M. at 1°C for 10 minutes. The clear supernatants were decanted and tested for their content of complement components by the method previously described (17).

The packed erythrocytes were then resuspended in 10 volumes of 0.9 per cent saline, chilled to 1°C, thoroughly mixed and again centrifuged. This washing was repeated three times. The saline washings, except for minute traces in the initial washing, contained no complement component activity and were discarded. The washed erythrocytes were made up to a volume of 40 cc. with 0.9 per cent saline, so that each cubic centimeter contained 1 unit of cells, 5 units of anti-sheep cell rabbit serum, and the combining components from 1 unit of complement.

1 cc. of each erythrocyte suspension was then incubated at 37°C for 30 minutes. If 10 per cent or less hemolysis occurred, a quantity of each specifically inactivated complement or individual component equivalent to the amount present in 1 unit of complement was added to 1 cc. of the red blood cell suspension, and incubated at 37°C for 30 minutes. The amount of hemolysis produced was estimated by comparison with a series of standard hemoglobin solutions and expressed as percentage of complete hemolysis. The same precautions expressed in previous publications (17) were observed in these experiments in order to obtain comparable and reproducible data. The temperature conditions were rigidly controlled. For the further clarification of the data, the per cent hemolysis resulting from the inter reactivations of the specifically inactivated complements is included in each protocol.

The Fixation of the Components of Complement by Sensitized Sheep Cells

In this experiment a comparison of the effects of normal complement and of specifically inactivated complements, as well as mid piece and end piece, was made by the separate addition of each to a volume of sensitized erythrocytes. The results obtained are summarized in Table I, and reveal the following facts: (1) All of C'4 and C'2 in normal serum was removed or inactivated by the sheep cell aggregates. (2) Apparently little C'1 and no C'3 of normal serum were fixed. (3) The inactivation of C'4 by hydrazine previous to fixation, while it did not markedly influence the combination of C'3

² Either untreated complement or specifically inactivated complements equivalent to quantities representing 40 units of fresh complement. All specifically inactivated complements which had been diluted during inactivation or separation were concentrated by lyophilization to their original volumes before employment in fixation experiments.

and C'2, resulted in an increase of the fixation of C'1, since upon the addition of end-piece only 40 per cent hemolysis occurred (4) The removal of C'3 by the insoluble carbohydrate prepared from yeast resulted in the almost complete fixation of the other components of complement (5) The inactivation of C'1 and C'2 by heat inhibited the fixation of C'4 (6) When end-

TABLE I

Packed sheep R.B.C. suspension —10 billion per cc	40 units —0.28 cc.— normal serum or treated serum	Anti-sheep red blood cell rabbit serum (1:20)	Hemolysis produced by supernatant after fixation	Hemolysis produced after addition of specifically inactivated complements					
				Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum treated with insoluble carbohydrate and hydrazine	Serum heated at 56° C for 30 min	CO ₂ -soluble fraction (end piece)	CO ₂ -insoluble fraction (mid piece)
cc		cc	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	Normal guinea pig serum	1	5	5	85	15	50	90	0
1	Serum treated with hydrazine	1	0	0	70	0	40	40	0
1	Serum treated with insoluble carbohydrate	1	0	0	10	0	tr	10	0
1	Serum treated with hydrazine and insoluble carbohydrate	1	0	0	10	0	10	10	0
1	Serum heated at 56°C for 30 min	1	0	90	90	60	0	0	0
1	End-piece	1	0	80	75	50	0	0	80
1	Mid-piece	1	0	0	50	0	0	tr	0
Specifically inactivated complements			Hemolysis						
			per cent						
Serum treated with hydrazine			0	0	85	0	90	90	0
Serum treated with insoluble carbo- hydrate			0	85	0	10	90	45	60
Serum treated with hydrazine and insol- uble carbohydrate			0	0	10	0	60	25	0
Serum heated at 56° for 30 min			0	90	90	60	0	0	0
End-piece			0	90	45	25	0	0	90
Mid-piece			0	0	60	0	0	90	0

piece which contained C'2, C'4, and a small amount of C'3, was added to sensitized sheep cell suspensions, little or no fixation of C'2 and C'4 occurred (7) Practically all of C'1 was removed or inactivated in the fixation of mid-piece, which apparently contained no C'2 or C'4 (8) C'4 did not combine in the absence of active C'1

These results are similar to those reported in an earlier publication (17), and show that the direct and immediate inactivation of whole fresh complement by fixation to sensitized red blood cells is due to a union of almost all of C'2 and C'4 and a part of C'1. As pointed out by Nathan (16) and ourselves (17), C'3 does not combine under these conditions.

The Nature and Role of the Fixed Complement Components

In order to learn whether the inactivation of complement during fixation is due to an actual adsorption of C'2-C'4 and C'1 and whether these components are functionally active after fixation, the resuspended cells were

TABLE II

1 cc. of 2.5 per cent suspension of sensitized R.B.C. which had previously been treated with	Hemolysis after incubation at 37°C. (control)	Hemolysis produced after addition of					
		Serum treated with hydrazine	Serum treated with insoluble carbhydrate	Serum treated with insoluble carbhydrate and hydrazine	Serum heated at 56°C. for 30 min.	CO ₂ -soluble fraction (end-piece)	CO ₂ -insoluble fraction (cold piece)
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Normal guinea pig serum	10	95	5	tr	25	5	30
Serum treated with hydrazine	0	0	0	0	0	0	0
Serum treated with insoluble carbhydrate	0	60	0	0	15	10	10
Serum treated with insoluble carbhydrate and hydrazine	0	0	0	0	0	0	0
Serum heated at 56°C. for 30 min.	0	0	0	0	0	0	0
End-piece	0	0	0	0	0	0	0
Mld-piece	0	0	0	0	0	0	0

treated as outlined in the section of this paper dealing with methods. The results are shown in Table II, and reveal the following —

1 The resuspended cells which contained the combining components of 1 unit of complement showed only 10 per cent hemolysis when incubated at 37°C for 30 minutes, indicating that although C'4 and C'2 and a small amount of C'1 had combined, another fraction of complement which did not combine is necessary for hemolysis. The addition of serum deprived of C'4 to the resuspended cells caused 95 per cent hemolysis, while the addition of serum deprived of C'3 yielded a hemolytic titer no greater than that of the resuspended cell control. C'3, therefore, although not fixed, is necessary for the final action of complement, in this case, hemolysis. Apparently, heating of serum to

plement produce changes in large quantities of substrate, in that complement is thermolabile, and in that the reaction is highly specific. He further points out that when the substrate is highly concentrated as compared to complement, the speed of the reaction is independent of the concentration of the unhemolyzed corpuscles. He points out that the one argument which has been advanced against classifying complement as an enzyme is the fact that complement and anti-sheep cell rabbit serum appear to obey the law of definite proportions. While the data presented here contribute little one way or another to the enzymic interpretation of C'1, C'2, and C'4, they appear to warrant the classification of C'3 as a catalyst, since this component is not fixed and is not used up in the process of hemolysis (13, 16, 20, 21).

Although Bessemans (22) asserts that mid-piece and end-piece are mutually supplementary, others claim that optimal hemolysis results when end-piece and mid-piece are present in the same proportions as in normal serum. Parsons (23) states that end-piece must be present in relatively high proportion, while mid-piece may be reduced considerably. Browning and Mackie (12) state that on increasing end-piece, a smaller quantity of mid-piece is needed for complete hemolysis. Since their experiments were reported before the discovery of C'4, the reason for these discordant results is apparent. It is evident from the experiments reported in the present paper that the activity of C'1 after fixation is pre-determined by the fixation of C'4. It is probable that when mid-piece contains C'4 together with C'1, it is to a certain degree supplementary to end-piece. Mid-piece devoid of C'4 is not supplementary and in fact may prove anticomplementary when used in excess, as has been frequently observed. This was clearly indicated in experiments on pure C'1 (24). In these instances, the adsorbed C'1 in the absence of C'4 may occupy all of the "key spots" (25) on the red cell-antibody complex and therefore prevent further combination by any C'4 molecules.

SUMMARY

- 1 At a temperature of 1°C, C'2, C'4, and C'1 combine with sensitized sheep erythrocytes, while C'3 does not combine.

- 2 C'1, although combining with sensitized cells in the absence of C'4, is hemolytically inert unless C'4 combines previous to, or simultaneously with it.

- 3 C'4 does not combine in the absence of C'1.

- 4 Although C'3 is not fixed by antibody-sheep cell aggregates, it is essential for hemolysis, operating on the sensitized red cell after the fixation of C'4, C'2, and C'1 and behaving as if it were a catalyst.

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SELECTIVE "STAINING" FOR ELECTRON MICROGRAPHY

THE EFFECTS OF HEAVY METAL SALTS ON INDIVIDUAL BACTERIAL CELLS

BY STUART MUDD, M.D. AND THOMAS F. ANDERSON,* Ph.D.

(From the Department of Bacteriology, The School of Medicine University of Pennsylvania, Philadelphia and the RCA Manufacturing Company Camden, New Jersey)

PLATES 11 TO 14

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Between the present lower limits of resolution of electron microscopy and of light and ultraviolet microscopy, are fine structures of great interest in almost every field of natural science, which have hitherto been inaccessible to direct observation. As examples of some of these structures currently under successful examination with the electron microscope, may be mentioned virus (1) and phage (2) particles, bacteria (3), antibody films (4, 5), the cuticle of insects (6), tracheae, tracheoles, and air sacs of insects (7), the iridescent scales of butterflies' wings (8), nerve and connective tissue fibrils (9), colloidal particles, smokes, fumes, plastics, pigments, insecticides latex, the surfaces of polished metals (10), and the silver grains on photographic plates (11).

The electrons emitted from the electron gun are subjected to an electromagnetic field which acts as a condensor lens and causes them to follow parallel paths in a narrow beam along the axis of the instrument. This beam passes through the object and thence through a narrow collecting aperture, through electromagnetic objective lens and projector lens, and finally to form the image on the fluorescent viewing screen or photographic plate. Electrons which pass through the object undeflected of course contribute to the brightness of the image, any electrons which are sufficiently deflected in the object so that they do not pass through the collecting aperture do not contribute to the brightness of the image. The image actually formed therefore is essentially a record of the amount of electron scattering from each point in the object. Points in the object appear dark in proportion to the number of electrons they deflect sufficiently so that they do not enter the collecting aperture.

The deflection or scattering of electrons of given velocity in passing through matter is primarily caused by interaction with the positive charge on the atomic nucleus and with the negative space charge of the satellite electrons. Both nuclear charge and number of satellite electrons are proportional to the atomic number of the element in question. The probability that electrons of given velocity will be deflected through a given angle in passing through the object will be proportional, to a first approximation, to the product of the density times the thickness of the object traversed or to the summation of the products of

* RCA Fellow on the Electron Microscope, National Research Council.

concentrations times atomic weights of the component atoms of the object multiplied by the thickness of the object. Although the mechanism of image formation is different, electron micrographs may therefore be considered analogous to x-ray pictures, since the darkness and brightness depend on the thickness and density of the specimen.

It follows immediately from these considerations that anything which increases the mean concentration or mean atomic weight of atoms in any part of the object will proportionately increase the darkness of the electron image of that part of the object. If heavy atoms can be attached selectively by chemical or other means to any part of an object, contrast in the image should be selectively altered and a result obtained equivalent to differential staining in an object viewed with visible light.

It has occurred to the senior author that the familiar heavy-metal germicides, as a class of reagents which do react selectively with certain components of protoplasm, have interesting possibilities in this connection. Fildes (12) has advanced evidence to indicate that the germicidal action of diffusible mercury salts in particular is attributable to their ability to form salts with sulfhydryl groups in the cell, heavy metal ions are known also to form salts, under proper conditions, with acidic groups of proteins.

Heavy metal salts have therefore been tried as a possible introduction to a future technology of selective "staining" for electron micrography. Although opportunity has not yet been afforded for systematic study of the factors involved, the results offered herewith show that selective increase in contrast, as a result of chemical action, is readily obtainable. An incidental finding of no little interest is that electron micrography affords a means of observing the action of at least certain classes of germicides on individual bacterial cells.

Materials and Technique

A strain of *Fusobacterium*, whose morphology under the electron microscope has been described (13), grown anaerobically on cysteine-ascitic fluid broth.

Eberthella typhosa (strain P115) grown on plain nutrient agar. Titration of this strain against O, O-H, and Vi¹ antisera gave strong agglutination with the O and O-H sera, but none with Vi antiserum. The sera of rabbits injected with P115 showed O and H agglutinins but no Vi agglutinin. Since strain P115 is an old laboratory strain it may be concluded that it has lost its Vi antigen.

Shigella dysenteriae and *Vibrio comma*, grown 22 hours on infusion agar slants. Both are old laboratory strains giving typical biochemical reactions, the question of their virulence has not been tested recently.

Stock solutions of heavy metal salts were prepared as follows. Silver nitrate dissolved in distilled water to make a 3 molar solution. The reaction of this solution was pH = 3.7. Lead acetate was dissolved in distilled water to make a solution of 0.59 M.

¹ For this serum we are indebted to Dr. Morton Klein of the Department of Public Health and Preventive Medicine.

concentration, pH = 5.6. Mercuric chloride was dissolved in distilled water to make a solution of 0.22 M concentration, pH = 3.5. These stock solutions were diluted as indicated in the text.

The bacteria were suspended in distilled water and placed in a droplet from a capillary pipette on a thin collodion film supported on a fine nickel screen. Difficulty has been encountered in earlier work in that any impurities dissolved or suspended dried around the object and obscured or complicated the picture. One of us has therefore introduced a "wash-off" technique.

The minute drop containing the specimens is placed on the mount and the latter is held in a forceps for about 30 seconds so as to allow some of the bacteria, (or other specimens), to settle on and adhere to the collodion film. The edge of the droplet on the mount is then touched to the meniscus of distilled water in a test tube held nearly horizontally. The droplet is violently propelled off the mount into the water by surface tension forces, leaving the sedimented bacteria on the collodion film relatively free of impurities. After this washing process has been repeated, the preparation is allowed to dry in air and introduced into the microscope.

In the present study this technique has been extended to allow the interaction of reagents, (germicides, immune serum), with the specimen. The droplet is placed on the mount; a droplet of the reagent on another capillary pipette is touched to the droplet containing the specimen at a given moment. Interaction and settling is allowed to proceed, if necessary in a moist chamber, for the desired time and the reagent is then washed away by touching the drop to a meniscus of water as described above. It is thus possible to follow as a function of time morphological changes produced in bacterial cells by chemical reagents or immune sera (14).

RESULTS

For the pictures that follow the germicidal solution and bacterial suspension were mixed in approximately equal volumes and allowed to interact for about 30 seconds. The acidity of the reagent solutions and osmotic influences may well have conditioned the results to a minor degree, but the striking effects obtained must have been caused primarily by the chemical reagents.

Fig. 1 shows cells of *Fusobacterium* dried from distilled water as a control. The protoplasm appears gray and fills most of the cells except for the tips in which the cell walls free of inner protoplasm can be seen (13). Fig. 2 shows cells of the same suspension of *Fusobacterium* after mixing with the 3 molar silver nitrate solution, (i.e., exposure to approximately 1.5 molar silver nitrate solution). The cell walls are unstained but the inner protoplasm is rendered opaque. Our interpretation is that the silver, as ions or otherwise, diffused rapidly through the cell wall and combined with the inner protoplasm.

Fig. 3 shows a cell of *Fusobacterium* after mixing with the 0.22 M mercury bichloride solution. The bacterial protoplasm is darkened and shrunk away from the cell wall. Fig. 4 shows *Fusobacterium* after mixing with 0.59 M lead acetate. Darkening is less apparent than with the silver or mercury salts, although the impression of coagulation and shrinkage of the protoplasm is even stronger than with the Ag and Hg salts.

Fig 5 shows cells of *Eberthella typhosa* dried from distilled water as a control. The flagella are uniform and distinct but afford relatively little contrast with the collodion film on which they are mounted. In Fig 6 are shown cells from a suspension of *E. typhosa* after mixing with 3 M AgNO_3 . The flagella have been completely destroyed and the protoplasm is black. The cell wall as in the case of *Fusobacterium* appears not to be "stained" by the silver. The entire cells are very much smaller than in the control, suggesting shrinkage.

Fig 7 shows cells of *E. typhosa* after mixing with 0.59 M $\text{Pb}(\text{Ac})_2$. The bacterial cells are swollen and protoplasm has escaped to form a diffuse halo about the cells. These swollen cells are an interesting contrast to the cells shrunk by exposure to AgNO_3 shown in Fig 6. Figs 6 and 7 are from the same bacterial suspension and at the same magnification. The flagella of the typhoid bacilli treated with $\text{Pb}(\text{Ac})_2$, (Fig 7), are darker than those of the control but are not perceptibly thickened. In the areas adjacent to the cells in which the flagella are surrounded by escaped protoplasm they appear to be bounded by a double dark line with a light axial line between them (Fig 7a), this is probably due to the accumulation of the dissolved protoplasmic components against the flagellum in drying of the preparation, and does not support the suggestion that flagella might be hollow tubules.

Fig 8 shows cells of *Shigella dysenteriae* suspended in distilled water for a few minutes and rapidly dried. The protoplasm fills the cell walls completely except in the case of one apparently partially cytolized bacterium. Areas of increased density are seen near the poles of the cells.

Figs 9 to 11 show cells of the same suspension of *Shigella* exposed for 30 seconds to 0.14 M (2 e isotonic) solutions, respectively, of AgNO_3 , $\text{Ni}(\text{NO}_3)_2$, and $\text{Pb}(\text{Ac})_2$. In the cells after exposure to AgNO_3 , (Fig 9), the protoplasm is darkened and in some cells shrunk away from the unstained cell wall. In Figs 10 and 11 the darkening of the protoplasm is in a number of cells limited to a part of the periphery of the inner protoplasm but is very intense.

Figs 12 and 13 show cells of *Vibrio comma* suspended and rapidly dried from distilled water. Fig 14 shows cells of this same suspension of cholera vibrios after 10 seconds exposure to $\text{Pb}(\text{Ac})_2$ in 0.29 M concentration. Close inspection of the cells shows a fine dark line bounding the protoplasm within the cells. This is evidently the protoplasmic membrane (15) or limiting surface of the inner protoplasm, stained by the penetrating lead. In some of the cells also exudation of the protoplasm as a result of injury may just be seen to be beginning. In Fig 15, which is another field of the same preparation as in Fig 14, there has been more exudation of protoplasm from injured cells.

The darkening and thickening of flagella and cell walls through combination with specific antibody to form antibody films have been demonstrated (5, 14). Chemical reagents selectively altering cell walls and flagella will also doubtless be found with further search.

SUMMARY

The physical basis of contrast and image formation in electron micrography is considered in relation to the possibility of recording selective chemical effects on cell components. A technology of selective microchemical analysis, equivalent to differential staining, is suggested as practicable in electron micrography.

Electron pictures of bacteria after exposure to salts of heavy metals have shown the bacterial inner protoplasm, but not the cell walls, to be selectively darkened, shrinkage, coagulation, or escape of protoplasm from the injured cells may result and be recorded in the electron micrographs.

Recording of the action of germicidal agents on individual bacterial cells is indicated as one promising field of application of microchemical analysis with the aid of the electron microscope.

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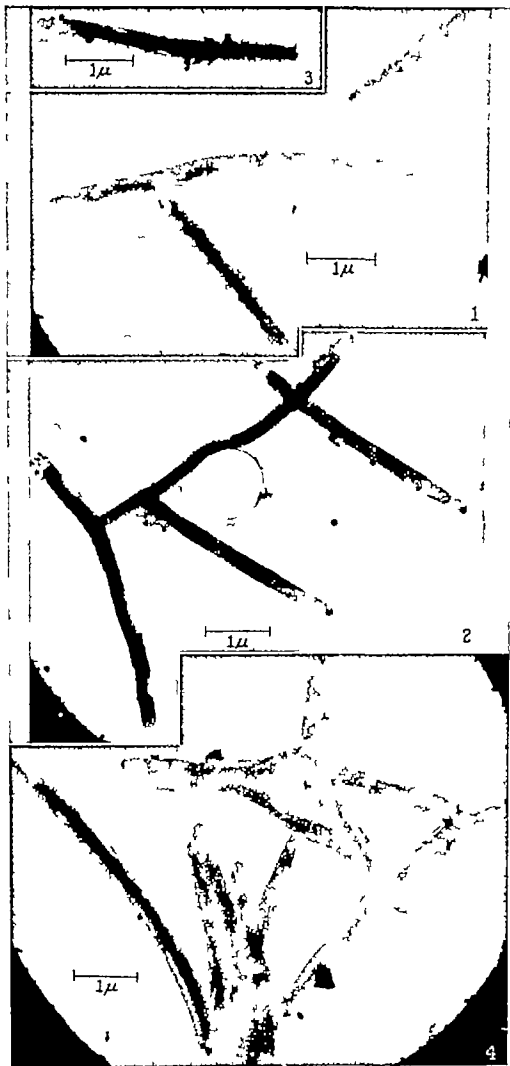
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The darkening and thickening of flagella and cell walls through combination with specific antibody to form antibody films have been demonstrated (5, 14). Chemical reagents selectively altering cell walls and flagella will also doubtless be found with further search.

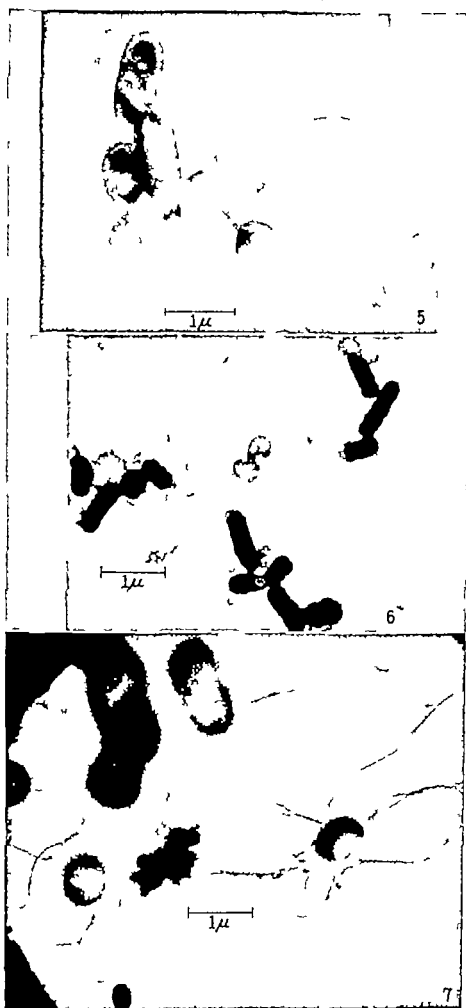


(Mudd and Anderson 'Selective staining' for electron microscopy)

EXPLANATION OF PLATES

PLATE 11

FIG 1	<i>Fusobacterium</i>	Cells dried from distilled water	Magnification	×
12,500				
FIG 2	<i>Fusobacterium</i>	Cells after exposure to 1.5 M AgNO ₃ solution		×
12,000				
FIG 3	<i>Fusobacterium</i>	Cells after exposure to 0.11 M HgCl ₂ solution		×
12,000				
FIG 4	<i>Fusobacterium</i>	Cells after exposure to 0.28 M Pb(Ac) ₂ solution		×
12,000				



(Mudd and Anderson Selective staining for electron microscopy)

PLATE 13

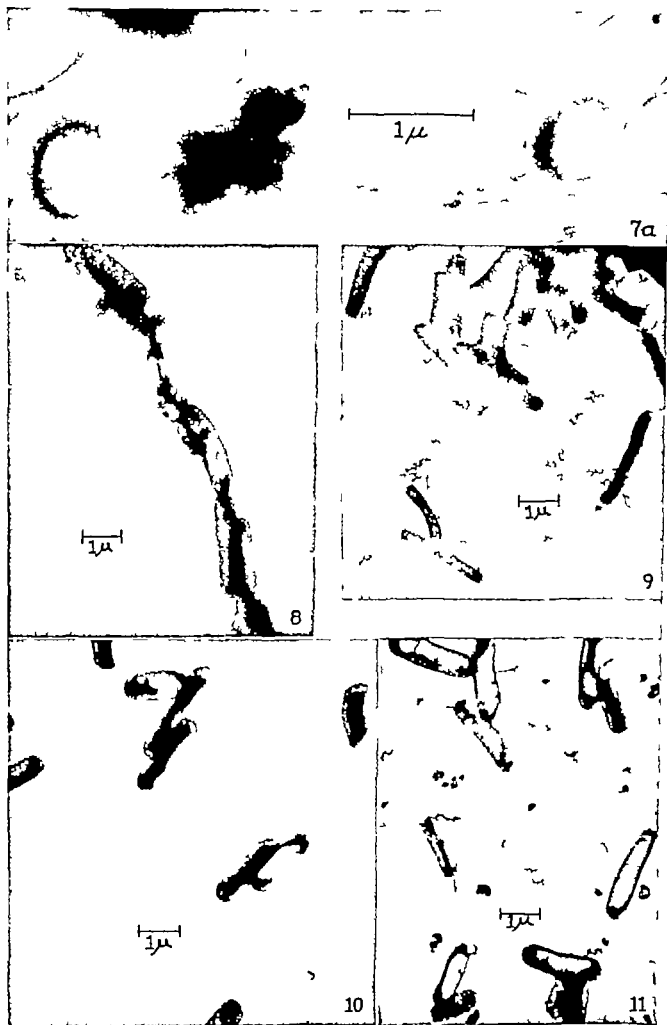
FIG 7a *Eberthella typhosa* Area of Fig 7 at higher magnification to show appearance of double, dark lines bounding flagella where they are surrounded by escaped protoplasm $\times 22,500$

FIG 8 *Shigella dysenteriae* Cells dried from distilled water $\times 7,000$

FIG 9 *Shigella dysenteriae* Cells after exposure for 30 seconds to 0.14 M Ag-NO₃ $\times 7,500$

FIG 10 *Shigella dysenteriae* Cells after exposure for 30 seconds to 0.14 M Ni(NO₃)₂ $\times 7,500$

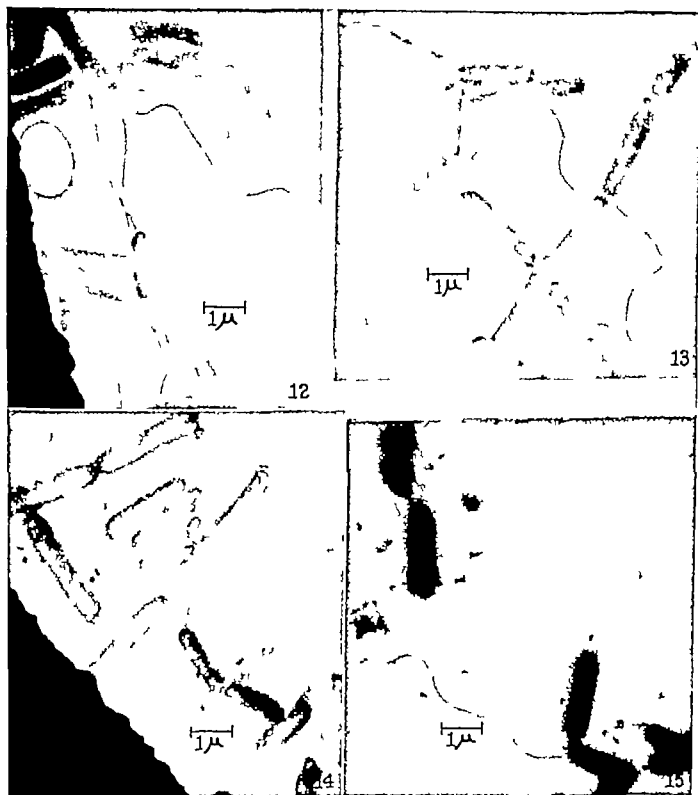
FIG 11 *Shigella dysenteriae* Cells after exposure for 30 seconds to 0.14 M b(Ac)₂ $\times 7,500$



(Mudd and Anderson Selective staining for electron micrography)

PLATE 14

FIG 12	<i>Vibrio comma</i>	Cells dried from distilled water	× 7,500
FIG 13	<i>Vibrio comma</i>	Cells dried from distilled water	× 7,500
FIG 14	<i>Vibrio comma</i>	Cells after 10 seconds' exposure to 0.29 M Pb(Ac) ₂	× 7,500
FIG 15	<i>Vibrio comma</i>	Cells after 10 seconds' exposure to 0.29 M Pb(Ac) ₂	× 7,500



A SEROLOGICAL CLASSIFICATION OF VIRIDANS STREPTOCOCCI WITH SPECIAL REFERENCE TO THOSE ISOLATED FROM SUBACUTE BACTERIAL ENDOCARDITIS

BY MATHILDE SOLOWEY*

(From the Department of Bacteriology, and the Delamar Institute of Public Health,
College of Physicians and Surgeons Columbia University, New York)

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The classification of the *viridans* streptococci has long been considered inadequate. Serological methods so far employed have shown them to be a heterogeneous group of organisms, while biochemical and cultural methods have yielded only a partially satisfactory species identification. Since these organisms are widely distributed on normal mucous membrane surfaces, it would help considerably in understanding the pathogenesis of infection in such a disease as bacterial endocarditis if the potential pathogens among the saprophytic forms of *viridans* streptococci could be identified. With this in mind a study of the group or type relationships among the *viridans* streptococci from several sources was undertaken.

Although the results fail to distinguish pathogenic forms in this group of bacteria, they offer a more exact method for the study of the epidemiology of infections with *viridans* streptococci.

The separation of all streptococci into hemolytic, *viridans* and indifferent forms as first evolved by Schottmüller (1) and subsequently worked out by Brown (2) has been a profound and useful means of differentiation. We know now, however, that it is not possible to draw such rigid lines between species of streptococci based upon their action on blood agar. It is well recognized, for example that some members of all the Lancefield groups appear as green producing streptococci and also that some strains among the so called *Streptococcus salivarius* or *Streptococcus faecalis* gave reactions on blood agar ranging from the typical alpha hemolysis to the gamma or anhemolytic type.

Furthermore, given *viridans* streptococci the subsequent differentiation of these into species offers great difficulties. The fermentation of certain sugars as well as the possession of one or more other biochemical properties has been utilized in attempts to classify these organisms. Sherman and his coworkers (3a-d) have in recent years made an exhaustive study of the *viridans* streptococci. By the application of several biochemical tests they have classified the *viridans* streptococci into three large species *Streptococcus salivarius*, *Streptococcus equinus* and *Streptococcus faecalis*. There is however considerable overlapping and the identification of a particular strain is frequently impossible.

Serological methods for classification have not thus far proven successful. At

* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science Columbia University

tempts at grouping by agglutination and agglutinin absorption, complement fixation, and precipitation have yielded disappointing results. Krumwiede and Valentine (4) found no relation between agglutinative and cultural characteristics of the *viridans* streptococci in a study of twenty-three colony strains from eight normal throats. Kinsella and Swift (5), in a study of twenty-eight strains using agglutination and complement fixation reactions, were unable to show correlation between the groupings determined by these methods and those determined by fermentation reactions. Furthermore, each streptococcus was antigenically distinct. Hitchcock (6a) corroborated the complement fixation results of Kinsella and Swift. With apparently no exceptions, all attempts to correlate antigenic strains by means of agglutination, agglutinin absorption, and complement fixation to either fermentative group, source of culture, morphology, or indeed any other property of the *viridans* streptococci have demonstrated the heterogeneity of these organisms.

In 1925 Lancefield (7a) reported on the immunological relationships of the *viridans* streptococci using purified chemical fractions of bacterial bodies. Under appropriate conditions, two different antibodies could be produced: one, an antibody for the bacterial protein (P) which showed group reactions with proteins from related species, and the other, an antibody for the non-protein, probably carbohydrate substance (S). This soluble specific substance was antigenic only when in the intact bacterial cell. The antibody for this was precipitated by the carbohydrate chemically extracted from the cell, and was closely related to specific agglutination of the homologous bacteria. When intact bacteria, then, were used for immunization, not only were specific antibodies produced, but also non-specific antibodies whose presence could be tested for by precipitation and complement fixation with protein fractions of the homologous and heterologous strains. Such P antibodies in serum might well be responsible for the non-specific cross-agglutination. The results that Lancefield obtained corroborated those of Kinsella and Swift (5) and Hitchcock (6b) in demonstrating group crossing among hemolytic and non-hemolytic streptococci and among streptococci and pneumococci as due possibly to the presence of similar protein fractions in these closely related species. These antigenic relationships were determined from a careful study of four strains of *viridans* streptococci isolated from bacterial endocarditis, although a few additional strains were tested to confirm the results.

Satisfied that the carbohydrate substance could be utilized to determine the antigenic specificity of the non-hemolytic streptococci, Lancefield branched from here to studies on the hemolytic forms. It was observed by Hitchcock (6a) and confirmed by Lancefield (7b, c) that the beta hemolytic streptococci also contained a carbohydrate "C" substance which was not type specific. That this "C" substance was believed to be identical in all hemolytic streptococci was accounted for by the fact that in these studies the strains were all from human sources. When streptococci from a wide variety of animal sources were studied it was observed that the "C" substance found in human strains was not present in strains from other sources. The possibility then presented itself that these organisms could be differentiated by means of similar "C" substances separate and distinct from those of human strains. This indeed has been established and is the basis for the Lancefield classification of the hemolytic streptococci. A concomitant stream of investigations on the antigenic mosaic of these organisms culminated in the work of Griffith (8). He demonstrated that with a rapid slide agglutination technique, the hemolytic streptococci from human infec-

tions could be subdivided further into twenty-seven types. These antigenic types were determined, as later revealed by Lancefield, by the presence of an acid-soluble protein component "M," separate and distinct for each type.

Since the hemolytic streptococci lend themselves to an orderly classification, based upon the presence of a "C" substance within the bacterial body, it might be reasonable to assume that the *viridans* streptococci containing similar "C" substances would also lend themselves to an orderly classification, although such a classification might reveal type rather than group relationships. It was believed that a reapplication of methods now carefully worked out for the hemolytic streptococci to a large number of *viridans* streptococci might indicate such group or type relationships.

Methods and Procedures

Strains of *viridans* streptococci were isolated from several sources (1) from blood cultures of patients clinically diagnosed as subacute bacterial endocarditis, (2) from human throats, and (3) from extracted teeth. The majority of endocarditis strains¹ were isolated within the last 3 years, although a few of them date back several years. Wherever possible, freshly isolated strains were used and tested as soon as they were made available in the laboratory. The *viridans* streptococci from human throats and extracted teeth were obtained for comparative purposes. The throat strains were isolated by picking typical single colonies from routine throat culture plates sent to the diagnostic laboratory of the Presbyterian Hospital, the extracted tooth strains² by streaking the tooth immediately upon extraction upon the surface of a blood agar plate. Typical single colonies were picked from the plates after incubation at 37°C. The reason for extraction, in most instances, was the presence of abscesses and a goodly proportion of these plates when so streaked showed almost pure cultures of green streptococci.

For further comparison a few strains of *viridans* streptococci from the vaginas of young infants and children were included.³ These strains were already isolated in pure culture.

Extracts of all strains of *viridans* streptococci were tested first against all the hemolytic group serums since it is known that some members of all the hemolytic groups may appear as green producing streptococci.

Immune serums were prepared against groups A, B, C, D, E, F, G, H, and K of the beta hemolytic streptococci. One culture⁴ in each group was used to immunize a

¹ *Viridans* streptococci from bacterial endocarditis were obtained from the diagnostic laboratory of the Presbyterian Hospital through the courtesy of Dr. F. B. Humphreys and Dr. H. M. Rose and from the diagnostic laboratory of Mt. Sinai Hospital through the courtesy of Dr. G. Schwartzman. Additional strains from this source were received from Dr. R. Lancefield, and from Dr. W. Tillett.

² The extracted teeth were obtained through the courtesy of the Division of Oral Surgery of the School of Dental and Oral Surgery, Columbia University.

³ *Viridans* streptococci from this source were very kindly supplied by Dr. G. Hardy.

⁴ The hemolytic streptococci used in preparing hemolytic group serums were obtained through the courtesy of Dr. R. Lancefield, Dr. G. Hobby, and Dr. J. Coffey.

pair of rabbits, except in groups B and D in which two cultures of different types were used. Serums were tested with at least one heterologous organism within the group as well as with members from all the other groups.

Eighteen strains of *viridans* streptococci freshly isolated from cases of bacterial endocarditis and nine strains freshly isolated from human throats and extracted teeth were selected arbitrarily. Two rabbits were immunized with each culture. Serums were tested with extracts of the homologous organism. When a satisfactory antibody response was obtained, as determined by a positive precipitin reaction, the serums were then tested not only with all the *viridans* streptococci but also with the available beta hemolytic streptococci from the several hemolytic groups.

The cultures of *viridans* streptococci were classified by their biochemical activities according to the method of Sherman and a comparison of these results with those obtained serologically was made.

Preparation of Immune Serum—Rabbits were immunized according to the method of Lancefield. The bacterial sediment from an 18 hour nutrient broth culture was suspended in 0.2 per cent formalin in saline and in 1/20 the volume of the original culture. The suspension was stored in the refrigerator in a tightly stoppered bottle, and was tested for sterility after at least 1 week. Immediately before use a 1:20 dilution of the formalized cell suspension was prepared.

Each rabbit received several series of injections. A series consisted of five injections of 1 ml. on consecutive days of 1 week followed by a week of rest. 5 days after the last injection of the second series and every series thereafter, test bleedings were made, serum collected, and stored in the refrigerator without a preservative. Many of the *viridans* streptococci gave a satisfactory antibody response after two or three series of injections.

Antigen Extract—The antigen extracts were prepared by a modification of the Lancefield technique. The bacterial sediment from 50 ml. of an 18 hour nutrient broth culture was suspended in 2 ml. of N/20 HCl in 0.85 per cent saline. The tube was then immersed in boiling water for 15 minutes, cooled under running water, and centrifuged. The supernatant was then removed and to it was added a drop of 0.04 per cent brom thymol blue. It was then neutralized with N/2 NaOH, centrifuged, and the sediment discarded. The remaining supernatant contained the antigen and was used in the precipitin tests.

Precipitin Test—The microtechnique was used for the precipitin tests. 0.04 ml. of serum was placed in specially tapered tubes, and 0.04 ml. of extract was layered over it. In some tests the sequence of delivery was reversed. The tubes were examined for ring formation after 30 minutes at room temperature, and again after 30 minutes at 37°C in the water bath. The contents of the tubes were then thoroughly mixed, placed in the refrigerator, and observed the next day, and then again after 48 hours, for precipitate or disc formation. The tests were carried out on undiluted extracts and serums. All serums were controlled by testing with the homologous organism and with a saline control.

Reactions were recorded as follows: ++, very strong positive, +, definite positive, ±, weak but definite positive, ?, questionable positive, —, negative.

More than 50 per cent of the tests were repeated two or three times for the serum.

of any particular trial bleeding, and at least two different HCl extracts were tested. The remaining tests were done only once with the serum giving the strongest positive reaction with the homologous strain. The individual serums of each pair of rabbits immunized with the same culture were tested with the homologous and representative heterologous strains. The tabulated data, however, record the results for the serum from each pair of rabbits which gave the stronger positive reactions.

Concomitant Biochemical Tests

Temperature Limits of Growth—These were determined in litmus milk. The cultures were adjusted to incubation temperature immediately after inoculation. The temperature limits tested for were 10°C and 45°C. All cultures were controlled by testing for growth in litmus milk at 37°C.

Thermal Resistance—5 ml. of skimmed milk were inoculated with 0.5 ml. of the test culture, and heated for 30 minutes at 60°C in a water bath.

Salt Tolerance Test—The ability to grow in media containing a high salt concentration was tested for by adding salt to 0.5 per cent glucose broth to make a final concentration of 6.5 per cent NaCl. Tubes of this media were inoculated with the test cultures and incubated at 37°C for at least 1 week before observations on growth were made.

Fermentation Reactions—These were observed in meat extract broth containing 1 per cent of the test sugar and 0.0007 per cent brom cresol purple. Tests were read after 4 days' incubation at 37°C.

Final pH—The final pH was determined in 1 per cent glucose broth. The tubes were incubated for 1 week at 37°C. Determinations were made with a glass electrode potentiometer.

All cultures were maintained in nutrient broth containing one drop of sheep's blood and were transferred monthly or bimonthly. Preliminary observations on macroscopic and microscopic morphology and on the appearance of the alpha type of hemolysis were made on all cultures.

RESULTS

Serological—A total of 108 strains of *viridans* streptococci from bacterial endocarditis and 99 strains from throats and extracted teeth were tested with serums prepared against the beta hemolytic groups A, B (two types), C, D (two types), E, F, G, H, and K. It was found that all except two failed to give precipitin reactions with any of these serums. Of the two exceptional cultures, one, SA5, from bacterial endocarditis, was a group G strain, the other, DSA34, from a human throat, a group K strain. Both strains appeared to give typical *viridans* reactions on sheep's blood agar. It is of interest to note that the two strains failed to give a precipitin reaction with any of the serums prepared against the endocarditis or throat and abscessed tooth strains.

Since SA5 which reacted with group G was isolated quite early in the study, this culture was used to immunize a pair of rabbits in order to test cross-reactions with group G cultures. This serum was difficult to prepare. SA5 grows

Precipitin Reactions of 107 Strains of viridans Streptococci Isolated from Bacterial Endocarditis*

		Extracts of <i>viridans</i> strains																																	
Group	Serum	SA 6†	SA 7	SA 8	SA 9	SA 11	SA 13	SA 16	SA 17	SA 27	SA 34	SA 40	SA 42	SA 45	SA 46	SA 48	SA 49	SA 52	SA 52	SA 107	SA 108	SA 109	SA 111	SA 112	SA 114	SA 115	SA 117	SA 119	SA 124	SA 123	SA 132	SA 135	SA 120	SA 129	
I	R57‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R65‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R68‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R93‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R82‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R90‡	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R1015	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
II	R60‡																																		
	R71‡																																		
	R85‡																																		
	R80‡																																		
	R1014																																		
III	R61‡																																		
IV	R63‡																																		
	R75‡																																		
V	R91‡																																		
VI	R77‡																																		
VII	R83‡																																		
VIII	R88‡																																		
IX	R1002§																																		
X	R1005§																																		
XI	R1007§																																		
XII	R1009§																																		
XIII	R1011§																																		
XIV	R1017§																																		
	R1003§																																		

Each serum was tested repeatedly with extracts from every strain, but only positive reactions are recorded

* Of these 107 strains, there were 33, extracts of which failed to react with any of the serums

‡ Indicates endocarditis serums serums prepared against strains isolated from bacterial endocarditis

Undaggered serums indicate those prepared against strains isolated from throats or extracted teeth

§ None of these serums reacted with endocarditis strains

|| +, ++, indicate reaction of serums with homologous strain

very lightly and quite slowly in nutrient, blood, or glucose broth so that the preparation of the culture for immunization was both tedious and difficult. Although a weak positive reaction appeared after the fourth series of injections, nevertheless several additional series were given in the hope that the antibody response might be increased. This was not found to be the case. Indeed the precipitin reaction appeared to become weaker in some of the later series. However the serums prepared against SA5 gave a weak but definite positive reaction with two group G cultures.

The *viridans* streptococcus DSA34, from a human throat, which fell into group K was not used for immunizing rabbits, since it was isolated in the latter part of the survey.

The results of the precipitin reactions of 107 strains from endocarditis and 98 strains from throats and extracted teeth with serums prepared against strains of streptococci from both sources are shown in Tables I and II, and Fig. 1. It is seen from Tables I and II that 14 serological groups have so far been established. 74, or 69.2 per cent, of the 107 endocarditis strains fall into one or more of 8 groups, and 33, or 30.9 per cent, cannot be grouped with the serums available. 62, or 63.2 per cent, of the 98 throat and extracted tooth strains fall into one or more of 9 groups, and 36, or 36.8 per cent, cannot be grouped with the serums now available. Since the throat and extracted tooth strains showed no significant differences among themselves in their group relationships or biochemical behavior, they have, for comparative purposes, been combined and considered here as one source.

Examination of Tables I and II reveals that group reactions are, on the whole, remarkably free from cross-reactions. Among the endocarditis strains (Table I) five—SA132, SA131, SA31, SA306, and SA30, gave evidences of some crossing. Despite the fact that SA132 and SA131 reacted with serums of groups VI and VIII respectively, they have nevertheless both been assigned to group I since they reacted with five and six serums, respectively, of the seven serums comprising group I. SA31, although it reacted with one serum (R1014) in group II, was likewise assigned to group I since it too reacted with five of the seven serums of group I. SA306 reacted with but one serum respectively in both groups I and II. Since there are as yet no instances of group II strains crossing with group I serums, and there is one instance of a group I strain crossing with group II (SA31), it seemed advisable at the moment, though perhaps questionable, to assign SA306 to group I. SA30 reacted with one serum of group IV, but since it reacted with four of the five serums of group II, it was assigned to group II. Among the throat and extracted tooth strains (Table II) two, CSA19 and DSA42, gave some evidences of crossing. Here too despite the fact that they reacted with groups V and XII respectively, since each reacted with six of the seven serums in group I, they both have been assigned to the latter group.

The reactions of serum R1003 (Table II) present a more difficult problem in allocation. Of the throat and extracted tooth strains reacting with this serum, six gave reactions with several serums of other groups, namely, one DSA53 with five serums of group I, the other five, DSA36, DSA20, DSA32, DSA3, and DSA6, with three or four serums of group II. The homologous strain CSA5 reacted only with the serum prepared against it (R1003). It might have been possible to assign this serum as another in group II. This seemed rather questionable since only five of the thirteen strains in group II gave reactions with this serum and since the homologous strain failed to react with group II serums or indeed any other serums. It seemed wiser to record the findings as such without making any definite assignment as to group.

Fig. 1 presents the break-down of endocarditis strains and throat and extracted tooth strains into their various serological groupings. Groups I, II, and IV contain *viridans* streptococci from both sources in approximately the same distribution. Groups III, V, VI, VII, and VIII contain only endocarditis strains, groups IX-XIV contain only throat and extracted tooth strains. Since approximately 50 per cent of the reacting strains from each source fall into groups I and II, the similarity among strains from both sources becomes evident, and strongly suggests that there is no essential difference between the *viridans* streptococci from each of these sources. It is indeed interesting to note that strains from both disease and non-disease sources are found to have similar group relationships.

In this connection it should be mentioned that a few strains of *viridans* streptococci, 15 in number, isolated from the vaginas of young infants and children, were tested with serums from groups I, II, III, and IV. All of these strains failed to react with any of the serums, with the exception of one strain which gave a weak positive reaction with two serums from group I.

Ten of the strains from endocarditis received from Dr. Lancefield had been maintained in the laboratory for several years, including among them several she herself used in her original work with the *viridans* streptococci (1925). Six of these strains reacted with endocarditis group serums, of which three fell into group I, two into group II, and one into group IV. This suggests that age does not change or destroy the antigenic structure of these organisms.

It must also be noted that among the 107 strains from endocarditis, five were represented in duplicate. The second culture of each pair was taken at intervals of 1 to 2 months after the first. They showed consistently similar pictures with the exception of one pair. That is, each of the duplicates of four pairs fell into the same group as its predecessor, the fifth pair did not do so. One member of the pair, the later culture, fell into group I, the earlier culture failed to react with any of the group serums.

Examinations of Tables I and II show that not all serums prepared against organisms of a single group give precipitin reactions with every member of that

group In Table III the individual serums of groups I and II are listed and the number of strains within these groups which react with each serum is indicated It is seen that the number of reacting strains within a group varies with the serum used For example, when serum R57 was used to test endocarditis strains, 40 of the 44 strains within the group gave positive reactions However, serum R1015, tested against the endocarditis strains, gave positive reactions with only 25 of the 44 strains within this same group The number of missed strains decreases when many serums within a group are used for

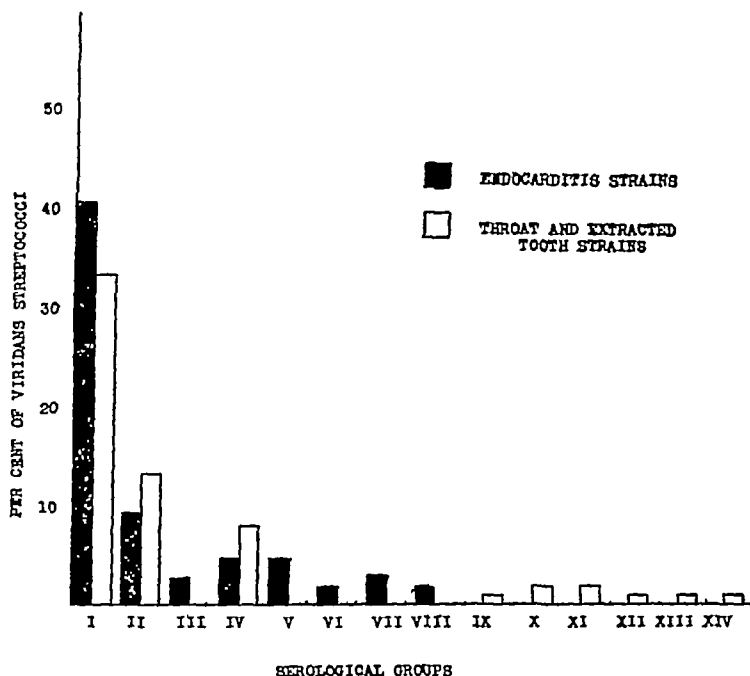


FIG 1 Per cent of *viridans* streptococci occurring in serological groups I-XIV

testing It should also be emphasized that groups I and II which contain the larger number of reacting strains from both sources were determined by serums prepared against 7 or 5 different strains, whereas the remaining groups (see Tables I and II) were determined by serums prepared against one or two strains Similar findings to these have been observed among the beta hemolytic group reactions Different organisms used for immunization may produce serums with varying breadth of antigenicity, thus possibly accounting to some extent for these variations in precipitin reactions The importance, therefore, of using as many strains as possible within a group for immunization becomes self-evident

Biochemical—A study of the biochemical activities of the *viridans* strepto-

cocci from bacterial endocarditis and throat and extracted teeth also was carried out. An attempt was made to correlate these findings with the anti-

TABLE III

The Number of Strains of viridans Streptococci Groupable by the Use of Individual Serums within Groups I and II

	Serum	Viridans streptococci from					
		Endocarditis			Throats and extracted teeth		
		No. +	Total + within group		No. +	Total + within group	
Group I	R57	40	of	44	30	of	33
	R65	39	of	44	29	of	33
	R68	34	of	44	27	of	33
	R93	30	of	44	26	of	33
	R82	28	of	44	19	of	33
	R90	27	of	44	20	of	33
	R1015	25	of	44	27	of	33
Group II	R60	9	of	10	10	of	13
	R71	9	of	10	8	of	13
	R85	8	of	10	11	of	13
	R80	8	of	10	10	of	13
	R1014	7	of	10	9	of	13

TABLE IV

The Relation of Serological Classification to Species Identification of Streptococcus viridans

		Viridans streptococci from			
		Endocarditis		Throats and extracted teeth	
		No	per cent	No	per cent
<i>Streptococcus salivarius</i>	Groupable strains	66	81.0	52	87.0
	Non-groupable strains	16	19.0	8	13.0
	Total strains tested	82	100.0	60	100.0
Undetermined species	Groupable strains	4	22.0	9	45.0
	Non-groupable strains	14	78.0	11	55.0
	Total strains tested	18	100.0	20	100.0
Total		100		80	

genic groups. It will be seen from Table IV, that of 100 strains from endocarditis submitted to the various biochemical tests suggested by Sherman *et al* for the differentiation of *viridans* streptococci, 82 or 82 per cent could be designated as *Streptococcus salivarius*. The remaining 18 per cent were com-

posed of a heterogeneous group whose demarcation into other streptococcus species was difficult to make. Of 80 throat and extracted tooth strains sub-

TABLE V

Relation of Biochemical Properties of Strains of viridans Streptococcus to the Serological Grouping

Source	Species		No of strains	Temperature limits of growth		Strains unable to resist 60°C 30 min No	Fermentations Lactose Salicin Raffinose Inulin Trehalose Mannitol Sorbitol No	Strains unable to grow in high salt concentration (6.5 per cent) No	Final pH 4.1-5.2
				No 45°C +	No 10°C +				
Endo-carditis	<i>Streptococcus salivarius</i>	Grouped serologically	66	49 17	0 66	64	63	66	All
		Ungrouped serologically	16	13 3	1 15	16	15	15	All
		Total	82	82	82	80	78	81	All
	Undetermined species	Grouped serologically	4	4 0	0 4	4	Variable	4	All
		Ungrouped serologically	14	8 6	2 12	13	Variable	12	All
		Total	18	18	18	17		16	All
Throats and extracted teeth	<i>Streptococcus salivarius</i>	Grouped serologically	52	41 11	0 52	48	50	51	All
		Ungrouped serologically	8	6 2	1 7	4	7	7	All
		Total	60	60	60	52	57	58	All
	Undetermined species	Grouped serologically	9	6 3	1 8	8	Variable	9	All
		Ungrouped serologically	11	7 4	3 8	6	Variable	7	All
		Total	20	20	20	14		16	All

jected to the same study, 60 or 75 per cent could be designated as *Streptococcus salivarius*, the residual heterogeneous group of 20 strains also giving difficulties in species identification.

Further analysis of Table IV shows that 81 per cent of the *Streptococcus*

salivarius strains from bacterial endocarditis are groupable with the serums now available, as compared with 87 per cent from throats and extracted teeth 19 per cent of the *Streptococcus salivarius* strains from bacterial endocarditis are unclassified serologically as compared with 13 per cent from throats and extracted teeth. Of the undetermined species among the endocarditis strains, 22 per cent can be grouped with the serums now available, 78 per cent cannot be thus grouped. Among the throat and extracted tooth strains which are species undetermined, 45 per cent can be grouped by the available serums, 55 per cent cannot be thus classified.

Table V shows the relation between antigenic groups and the various biochemical properties exhibited by the *viridans* streptococci. There is no obvious correlation between any particular biochemical property and the serological grouping, although the similarity of strains from both sources is apparent. Furthermore the distribution of *Streptococcus salivarius* strains, which constitute the large body of identified organisms, parallels rather closely the numerical distribution of strains within each serological group. The confusion encountered in allocating any particular strain to a species by means of the determinative tests employed for species identification, cannot be over-emphasized. Fermentation reactions are known to be variable. The other diagnostic properties here used, thermal resistance, temperature limits of growth, ability to grow in media containing a high salt concentration, were also found to be variable. A particular strain by virtue of the results of some of the tests might be classified within a definite species, but the results of the remaining tests might or might not be corroborative. Such a comparative study of the biochemical and immunological behavior of the *viridans* streptococci reveals once again the greater definitive value of serological methods in breaking down what at one time might have been considered a homogeneous species into a number of distinct immunological groups.

DISCUSSION

It is apparent from the data presented that the *viridans* streptococci are amenable to classification by serological methods. A total of 207 strains of *viridans* streptococci from several sources were subjected to analysis. Two strains fell into groups G and K respectively of the beta hemolytic groups. 136, or 66 per cent, of the remaining 205 strains could be classified into 14 serological groups. The justification for any classification, however, depends entirely upon the use to which it is put. At the inception of this study the hope was expressed that some correlation might be made between the classification of the *viridans* streptococci and their potential pathogenicity. The fact is, however, that it is here revealed that we cannot as yet make any distinction between organisms implicated in bacterial endocarditis and those found in abscessed teeth, or even in those present normally in the human throat. This, of course, does not necessarily imply that there is no distinction but rather

that we cannot so far by our methods determine its presence. Conversely should there actually be no differences among these organisms the problem becomes highly significant. The reason then that the *viridans* streptococci are so often associated with bacterial endocarditis may be due entirely to the fact that these organisms are so widely prevalent in the mouth. The ease with which they can invade the blood stream has been amply demonstrated by several investigators (9-16). A presumptive causal relationship between the teeth as a precedent focus of infection and subacute bacterial endocarditis has gained emphasis in recent years (13, 17-24). Instances have been cited of from one to as many as thirteen cases in which subacute bacterial endocarditis seemed to follow in almost direct relationship within a few days or weeks after dental extraction in septic mouths in individuals with precedent cardiac abnormality. The problem of traumatic injury becomes of paramount importance. If we cannot distinguish pathogens or presumable pathogens among the saprophytes, then any organism once it breaks the barrier into the blood stream can presumably be the causative agent in this disease process.

The question of reinfection or multiple infection in bacterial endocarditis is now amenable to study. Five cases in this study are represented by duplicate cultures, in four, the same serological type was obtained on both occasions. In the fifth case, the evidence indicates that two different serological types were present. Such a group of cases is too small in number to be evaluated statistically, thus the fifth case must assume its own significance. The possibility of reinfection with an entirely different serological type cannot be overlooked, and, indeed, may be more usual than these numbers seem to indicate.

That there may, however, be differences among the *viridans* streptococci relating to source of culture cannot be dismissed entirely. It will be remembered that a few strains, fifteen in number, from the vaginas of young infants and children, were tested with serums from four of the *viridans* groups. With the exception of one strain which reacted with group I serums, none of the remaining strains reacted with these serums. Although the number of strains tested are small, there is some suggestion that the *viridans* streptococci from this source may be different serologically. Such a possibility requires further investigation.

It would seem from the evidence presented here, that the serological identity of a strain in any particular case may be determined, and its relationship to a possible focus of infection more easily followed. That the presumable pathogens among the *viridans* streptococci cannot be distinguished readily is not an unexpected finding, since it is more than likely that any *viridans* streptococcus given the necessary conditions, can initiate bacterial endocarditis. However, the validity of a classification of these organisms cannot be dismissed. It remains for further study to bring out the uses to which such a classification may be put.

SUMMARY

A total of 108 strains of *viridans* streptococci from subacute bacterial endocarditis, and 99 strains from human throats and extracted teeth have been studied, and approximately two-thirds of all the strains so far have been differentiated into a number of serological groups. The similarity between strains from these sources is evident from the fact that approximately 50 per cent of the reacting strains from each source fall into two groups I and II, and that three groups I, II, and IV, contain streptococci from both sources in approximately the same distribution. Fifteen vaginal strains of *viridans* streptococci failed, with the exception of one strain, to react with groups I-IV of the endocarditis serums. More than three-fourths of the strains from both sources were *Streptococcus salivarius* as determined by Sherman's criteria. However, no correlation between the biochemical and serological classification could be made.

It may be concluded that the *viridans* streptococci are amenable to classification by serological methods. The results so far obtained do not indicate a serological difference between strains of *viridans* streptococci isolated from subacute bacterial endocarditis, and those isolated from human throats and extracted teeth. The greater definitive value of serological methods in the identification of a particular strain is indicated.

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STUDIES IN RODENT POLIOMYELITIS

V INTERFERENCE BETWEEN MURINE AND MONKEY POLIOMYELITIS VIRUS*

By CLAUS W JUNGBLUT M.D., AND MURRAY SANDERS M.D

(From the Department of Bacteriology College of Physicians and Surgeons, Columbia University New York)

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The occurrence of synergism or antagonism between microorganisms or viruses is generally known to workers in the field. Even though the mechanism of these phenomena is by no means well understood, the fact that the course of an infectious disease may be significantly altered by the concomitant action and interplay of two different etiological agents, or their growth products, offers an attractive field for experimental investigation.

As far as bacterial antagonism is concerned, inhibitory effects are usually the result of a destructive action *in vitro* and *in vivo* of certain bacterial enzymes, like pyocyanase, gramicidin, and penicillin, upon certain microorganisms. Among the protozoa, a well defined mutual suppression of two invading parasites has been described for mixed infections of bartonella and eperythrozoon in mice (1). However, not until one comes to the field of virus diseases is interference found firmly established as a distinct biological phenomenon. Probably the first reference to domination of one virus by another was made in 1929 by McKinney (2) who reported that a yellow mosaic virus, derived from the common light-green mosaic of tobacco, would not propagate in tobacco plants in which the common-mosaic virus was already present. Subsequent studies by McKinney (3) and others (4) have widened the scope of the interference phenomenon among plant viruses and added much to our knowledge of how to utilize this reaction as a possible means for establishing relationships between mutants and non-relationships between distinct viruses.

Examples of interference in virus infections of animals and man are not very numerous, nor have those on record received more than scant attention. What appears as evidence of crossed resistance has been described for certain virus systems, i.e. pseudorabies-virus B (5) and vaccinia herpes (6), but such protection as may be observed in these cases is probably due to an overlapping group immunity rather than to any immediate reaction between the viruses themselves. Other instances, however, may be quoted in which interference seems to occur because of mutual interaction, direct or indirect, between the

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opposing viral agents. Thus, a peculiar type of resistance to superinfection has been recorded for both encephalitogenic and non-encephalitogenic strains of herpes virus in that a second intracerebral dose, following shortly after a preliminary corneal or intradermal dose, causes a mutual extinction of the effects of the two injections (7), similarly, it is said that if rabbits receive a series of intravenous injections of fixed virus after subdural infection with street virus, no rabies develops (8). Furthermore, Hoskins (9) found that intramuscular injection of a neurotropic strain of yellow fever virus, which is usually harmless for monkeys, protects these animals against simultaneous infection with a highly pathogenic viscerotropic strain of the same virus. Subsequently, Findlay and MacCallum (10) showed that the injection of a mixture of Rift Valley fever and yellow fever virus into *rhesus* monkeys served to save a majority of the animals from death by yellow fever infection, conversely, a single inoculation of mice with neurotropic yellow fever virus and pantropic Rift Valley fever virus definitely protected a few mice against the latter disease and delayed the death of others. A well marked sparing effect of the virus of lymphocytic choriomeningitis upon poliomyelitic infection in monkeys has also been described by Dalldorf and his associates (11). In all these cases interference takes place with such rapidity, and the resulting protection is limited to such brief intervals, that the failure of infection can hardly be ascribed to forces of acquired immunity, as generally denoted by this term. Moreover, in the last two instances, antagonism occurs between serologically unrelated viruses, a fact which would tend further to minimize the involvement of any specific immunological effects.

No explanation can be given, at present, for these protective phenomena. Above all, it is uncertain whether one virus acts directly upon the other so as to produce complete annihilation of both, or whether the protection is due to a restriction of viral propagation in selective cell territories which are shared by the two infectious agents. Finally it is conceivable that one virus may elaborate a soluble substance which checks the growth of the other virus. Such inhibitory substances, derived from and acting against the same virus, have already been demonstrated in tumor tissue of the Rous chicken sarcoma (12).

It will be recalled that a powerful antagonism between the murine strain of SK poliomyelitis virus and poliomyelitis monkey virus (SK and Aycock strains) was discovered previously in the course of this work (13). Further progress with this problem has recently been reported (14, 15). It is the object of this communication to present in detail the experimental basis on which these observations rest and to record the results that can be obtained in monkeys, infected with poliomyelitis virus, by administering murine virus at various stages of the disease.

EXPERIMENTAL

The experimental work is presented in three sections. The first section deals with the results obtained by the inoculation of monkeys with mixtures *in vitro* prepared of murine and poliomyelitis virus, the second with attempts to protect monkeys against poliomyelitic infection by prophylactic administration of murine virus, the third with efforts to block the course of poliomyelitis in monkeys by injecting murine virus at certain intervals following infection with monkey virus.

Results Obtained with the Intracerebral Injection of Mixtures of Murine and Poliomyelitis Virus into Rhesus Monkeys

Mixtures were prepared by combining 0.5 cc. of murine virus suspension (obtained from the brain of mice paralyzed by SK murine poliomyelitis virus) with 0.5 cc. of monkey virus suspensions (obtained from the cord of monkeys paralyzed by monkey passage poliomyelitis virus). Swiss mice, 12 to 15 gm., and *rhesus* monkeys, 1800 to 2500 gm. were used throughout this work. Immediately after their preparation these mixtures in a volume of 1 cc. were injected intracerebrally into monkeys. Since earlier experience with tissue culture murine virus (15) had suggested that the effectiveness of interference between the opposing viruses may depend upon certain quantitative relationships, murine and monkey virus were employed in graded doses. The strains of monkey virus used in these experiments were the Aycock and the RMV virus. Tests with SK monkey poliomyelitis virus were omitted, partly because effective interference by mixing SK murine virus (mouse or culture virus) with SK monkey virus had previously been described (13, 15), and, partly because considerable difficulties were encountered in maintaining the SK strain of poliomyelitis virus at a uniformly high level of virulence in serial passages through monkeys. The specificity of the interference was determined by injecting intracerebrally into monkeys control mixtures consisting of poliomyelitis monkey virus in combination with (1) saline, (2) normal mouse brain suspensions, (3) murine virus brain suspension inactivated by heating for $\frac{1}{2}$ hour at 75°C., and (4) herpetic (L.F. strain of herpes virus) mouse brain suspension. The results of these tests are brought together in Tables I and II.

It will be seen from Table I that interference between murine virus and Aycock monkey virus occurred regularly at levels of 1:10 dilution of monkey virus and 1:10 dilution of murine virus, the same dose of murine virus interfered effectively with all higher dilutions of monkey virus, except a dilution of 1:500.¹ In some monkeys a transient weakness was observed after injection with mixtures of the two viruses, particularly those containing an excess of

¹ Brain and cord of this monkey when sacrificed at the height of paralysis, failed of transmission to monkeys with the production of paralysis, whereas transfer to mice induced paralysis. The paralysis in this monkey therefore may have been caused essentially by murine virus activity.

murine virus, this condition was probably caused by mouse virus activity. It will further be noted that the effectiveness of interference is gradually lost when successive dilutions of murine virus are combined with a constant dose of Aycock virus. Thus, of three monkeys receiving mixtures of Aycock virus 1:10 and murine virus 1:100 one animal developed paralysis, of three monkeys receiving mixtures of Aycock virus 1:10 and murine virus 1:1000 two animals became paralyzed, whereas neither of two monkeys injected with mixtures of

TABLE I
Interference between Murine Virus and Monkey Virus (Aycock Strain) in Mixture Experiments

Monkey virus 0.5 cc.	Murine virus 0.5 cc.	Result		
		Complete paralysis	Partial paralysis	No paralysis
1:10	1:10	0	0	1
1:100	"	0	0	1
1:500	"	1	0	0
1:1,000	"	0	0	1
1:10	1:10	0	0	2
"	1:100	1	0	2
"	1:1,000	1	1	1
"	1:10,000	2	0	0
	Controls			
1:10	Saline	1	0	0
1:50	"	2	1	0
1:10	1:10 (normal mouse brain)	1	0	0
1:100	" "	1	0	0
1:500	" "	0	1	0
1:1,000	" "	1	0	0
1:10	1:10 (heated murine virus)	1	0	0
1:10	1:10 (herpetic mouse brain)	1	0	0

Aycock virus 1:10 and murine virus 1:10,000 escaped the disease. Since all control monkeys injected with doses of Aycock virus ranging from 0.5 cc of a 1:10 to a 1:1000 dilution succumbed to paralysis it appears that 0.5 cc of a 1:10 dilution of murine virus was capable of protecting against at least 100 minimal paralytic doses of poliomyelitis virus. Normal mouse brain and herpetic mouse brain exercised no protective action and the interfering principle in murine virus was evidently destroyed by heating for $\frac{1}{2}$ hour at 75°C.

An inspection of Table II shows that similar interference could be obtained between murine virus and RMV monkey virus, except that the lowest level of

effective interference began with a combination of RMV virus diluted 1:100 and a dilution of 1:10 murine virus, the same dose of murine virus protected effectively against all higher dilutions of RMV virus. When one estimates the degree of effectiveness of this interference and considers the fact that paralysis occurred in control animals injected with RMV virus dilutions up to 1:10,000, simple calculation shows that a dilution of murine virus of 1:10 was capable of protecting once more against at least 100 minimal paralytic doses of poliomyelitis virus.

The above findings, obtained with two different strains of monkey poliomyelitis virus, suggest that the interference between murine virus and monkey

TABLE II

Interference between Murine Virus and Monkey Virus (RMV Strain) in Mixture Experiments

Monkey virus 0.5 cc.	Murine virus 0.5 cc.	Result		
		Complete paralysis	Partial paralysis	No paralysis
1:10	1:10	1	0	0
1:100		0	0	1
1:200		0	0	1
1:500		0	0	1
1:1,000		0	0	1
1:5,000		0	0	1
1:10,000		0	0	1
1:200	Controls Saline	1	0	0
1:500		1	0	0
1:1,000		0	1	0
1:5,000		1	0	0
1:10,000		1	0	0

virus proceeds on some quantitatively fixed basis. No precise formulation can be offered at this time of the actual quantitative relationships involved since virulence titrations of the two strains of poliomyelitis virus were not carried to their respective end points. However, the available data indicate that 0.5 cc. of a 1:10 dilution of murine virus will counteract at least 100 minimum paralytic doses of poliomyelitis virus, irrespective of the strain used, when such mixtures are injected intracerebrally into *rhesus* monkeys. Upon injection of apparently balanced virus mixtures not much propagation of either virus seems to occur. Thus, on one occasion in which a monkey was sacrificed on the 12th day following intracerebral injection of a non pathogenic mixture of the two viruses, only traces of murine and of poliomyelitis virus could be recovered from brain, cord, or spleen, as determined by transfer of these tissues to mice and monkeys. Symptomless survival of monkeys following intracerebral

injection with mixtures of murine and poliomyelitis virus rarely seems to induce any permanent immunity. Thus, 4 of 5 such monkeys developed prostrating paralysis upon reinfection, 1 month later, with Aycock virus.

Results Obtained with the Administration of Murine Virus in Monkeys before Infection with Poliomyelitis Virus

At various intervals before intracerebral infection with poliomyelitis virus (RMV, Aycock) monkeys were given murine virus by the intravenous route. The murine virus was prepared by grinding three infected mouse brains in 9 cc. of tissue culture murine virus fluid, to yield a 10 per cent virus suspension. The suspension was allowed to settle for about 5 to 10 minutes and the turbid supernatant was used in amounts of 6 to 8 cc. for one intravenous dose; these injections must be given very slowly in order to avoid shock. Murine virus was given in repeated doses, varying from 3 to 5 injections, each dose being administered on successive days, the interval between the last injection of murine virus and poliomyelitic infection extended from 2 weeks to 1 day. One prophylactic series of murine virus injections, in some cases, constituted the only mode of treatment, in other cases, multiple injections of murine virus were resumed at irregular intervals following infection with poliomyelitis virus. A total of five experiments were run, in three experiments infection was produced by RMV virus² and in two by Aycock virus. Each experiment contained a variable number of treated monkeys and an adequate number of untreated controls, all of which were infected with the same dose of poliomyelitis virus. The results of the five experiments are listed in Table III which also gives all details concerning technical procedures.

The results obtained in the five different experiments, as given in Table III, are not strictly comparable, inasmuch as the experimental conditions varied considerably from test to test. However it appears that in the first three experiments, in which RMV virus was used, there were 3 monkeys in a treated group of 14 animals which remained entirely free from symptoms and 2 additional monkeys which developed atypical paralysis after greatly prolonged incubation periods. By contrast, all of 8 accompanying controls succumbed promptly to the disease. In the next two experiments in which Aycock virus was used a treated group of 12 monkeys included 10 animals which failed to show any paralytic symptoms whatsoever and 1 monkey which developed delayed paralysis, whereas all of 11 accompanying controls succumbed to the disease in a typical manner. In the group of treated animals which had failed to develop paralysis 6 monkeys survived sufficiently long to be reinfected, 4 weeks later, with Aycock virus. All 6 monkeys proved fully susceptible to reinfection.

In evaluating the mechanism of protection in these experiments an impres-

² These experiments include 14 monkeys to which reference was made in an earlier paper (16).

TABLE III		Interference between Murine Virus and Monkey Virus in Prophylactic Experiments	
	Mode of murine prophylaxis	Infection with monkey virus	Result
1	Subcutaneous	Subcutaneous	Interference
2	Intraperitoneal	Intraperitoneal	Interference
3	Intramuscular	Intramuscular	Interference
4	Intranasal	Intranasal	Interference
5	Intracerebral	Intracerebral	Interference
6	Intracerebral	Intraperitoneal	Interference
7	Intraperitoneal	Intracerebral	Interference
8	Intramuscular	Intracerebral	Interference
9	Intranasal	Intracerebral	Interference
10	Intracerebral	Intracerebral	Interference

TABLE III

Experiment No	No. of monkeys	Mode of murine prophylaxis		Infection with monkey virus		Result		
		Injection of murine virus	Interval between prophylaxis and infection	Strain	Dose	Complete paralysis	Partial paralysis	No paralysis
I	2 murine 2 controls	5 injections before infection	2 wks.	RMV	0.5 cc. 1:10	1 (9 days)		
II	7 murine 3 controls	5 injections before infection	1 wk.	"	" " "	2 (9-11 ") 2 (6 ")	1 (9 days)	0
III	3 murine 3 controls	5 injections before infection	—	RMV	0.5 cc. 1:100	3 (9-12 days)	2 (10-12 days)	0
IV	3 murine 3 controls	5 injections before infection	5 days	RMV	0.5 cc. 1:200	3 (5-6 ")	0	2
	3 murine 3 controls	3 injections before infection 3 injections before and 4 injections after infection	1 day 1 "	Aycock "	0.5 cc. 1:50 "	3 (7-11 ") "	0 0	1 0
V	6 murine 3 controls	3 injections before and 4 injections after infection	1 day	Aycock	0.5 cc. 1:50	1 (20 days)	2	3
	6 murine 3 controls	3 injections before and 4 injections after infection	1 day	"	"	1 (7-8 days) 1 (11 days)	1 (11 days)	0
Totals	26 murine 19 controls	—	—	" " "	" " "	8 (8-12) 9 18	4 1	13 0

* This interval denotes the time elapsed between the last injection of murine virus and infection with monkey virus.

				9 18	0 4 1	0 13 0
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* This interval denotes the time elapsed between the last injection of murine virus and infection with monkey virus.

propagates itself in the monkey only to a limited extent and is ordinarily ex-
creted within 1 or 2 weeks. In the light of these considerations the greater
success which attended the Aycock experiments as compared with the RMV
experiments should cause no surprise. In the first place, the dose of Aycock
virus used was not of overwhelming virulence, furthermore, some of the
treated animals received additional injections of murine virus after infection
with poliomyelitis virus finally, the interval which separated the last prophylaxis

lactic injection of murine virus from the date of poliomyelitic infection was not over 1 day. Obviously, in all these respects, the experimental conditions were much severer in those experiments in which RMV virus was used. It remains to be seen whether equally good results can be obtained with both strains of poliomyelitis virus, provided adequate allowance is made for dosage of monkey virus, dosage of murine virus and, particularly, a more favorable spacing of intervals between injection of the two viruses.

Results Obtained by the Administration of Murine Virus to Monkeys Following Infection with Poliomyelitis Virus

The experiments described in this section were undertaken in order to determine whether the injection of murine virus in monkeys, subsequent to infection with poliomyelitis monkey passage virus, was capable of modifying or blocking the course of the disease.

Three strains of monkey virus were employed in this work, *i.e.* the SK, the Aycock, and the RMV virus. Infection with monkey virus was produced by intracerebral injection. Following infection, certain intervals were allowed to elapse before the introduction of murine virus by the intravenous route, these intervals varied from a few minutes (1st day of infection) to 96 hours (5th day of infection). The infecting doses of Aycock and RMV virus, respectively, represented at least 20 to 50 minimal paralytic doses for each strain, *i.e.* Aycock 0.5 cc 1:10 to 1:50 dilution and RMV 0.5 cc 1:10 to 1:200 dilution, SK monkey virus was used in amounts of 0.5 cc 1:10 to 1:100 dilution, doses which fell within the approximate range of virulence of this particular strain. The dosage of murine virus varied widely from test to test. While no attempt was made to determine the minimum amount of murine virus which would afford protection at various intervals, some monkeys in which treatment was begun on the day of infection received one single injection only of murine virus. On the other hand, all monkeys in which treatment was delayed beyond the 1st day of infection were subjected to a series of repeated injections (5 to 9 injections). Such injections always extended over a period of several days (1 to 5 days), in some cases, two injections were given each day, in others only one dose of murine virus was administered daily. The murine virus was prepared in the same manner as previously described for the prophylactic experiments, *i.e.* a single dose consisted of three infected mouse brains, ground in 9 cc of undiluted tissue culture virus fluid, so as to yield a final virus concentration of 1:10. Each experiment included a variable number of animals under treatment with murine virus and an adequate number of control animals. The control animals were infected with the same dose of monkey virus and, for the greater part, remained entirely free from any form of control treatment, in one experiment the controls received an equal number of intravenous injections of normal mouse brain, suspended in uninoculated tissue culture fluid instead of murine virus preparations. Three experiments were carried out with SK virus, three with RMV virus, and six with Aycock virus. The results obtained are given in Tables IV, V, and VI.

The data shown in Table IV indicate that monkeys which had received murine virus were well nigh completely protected against poliomyelitic infec-

tion induced by SK virus. This protection extended practically undiminished from the 1st day of the infection to the 96 hour interval. Thus, among a total of 23 monkeys, treated between the 1st and 5th day during the incubation period of the disease, 20 remained entirely free from paralysis, whereas of a total of 11 controls only 2 escaped paralysis. Unfortunately, the SK monkey virus in these tests failed to paralyze all of the control animals, moreover, in some of the paralyzed controls paralysis was only partial. Such marked fluctuations in the virulence of the SK monkey virus, in our experience, have

TABLE IV

Interference between Murine Virus and Monkey Virus (SK Strain) in Therapeutic Experiments

Experiment No.	No. of monkeys	Infection with monkey virus Dose	Mode of murine therapy		Result		
			Interval between infection and therapy	Injections of murine virus	Complete paralysis	Partial paralysis	No paralysis
I	1 murine	0.5 cc. 1:100	No interval	1	0	0	1
	6	" "	5th day of disease	5-8	0	1	5
	4 controls	" "	—	—	2	2	0
II	2 murine	0.5 cc. 1:100	No interval	1	0	0	2
	4	" "	3rd day of disease	5-6	0	1	3
	4 " "	" "	5th " "	5-6	0	0	4
	4 controls	" "	—	—	1	2	1
III	3 murine	0.5 cc. 1:10	3rd day of disease	5-7	0	1	2
	3	" "	5th	7	0	0	3
	3 controls	" "	—	—	0	2	1
Totals	23 murine				0	3	20
	11 controls				3	6	2

been typical of this strain of virus and render it unsuitable for critical tests. The available evidence, therefore, while suggesting that a definite therapeutic effect had been achieved, could not be regarded as entirely conclusive.

A more clear-cut picture is presented by the data given in Table V, which lists the results obtained in monkeys infected with RMV virus. It appears that of a total of 20 monkeys, treated with murine virus between the 1st and 5th day of the incubation period, 7 remained entirely free from paralysis, whereas all 9 accompanying controls succumbed to the disease. It will also be observed that 4 additional treated animals recovered with partial paralysis, on the other hand, no control animal survived the prostrating paralytic attack which is characteristic of the RMV strain. Treatment with murine virus had therefore undoubtedly afforded a considerable degree of protection against poliomyelitic infection induced by the RMV strain, particularly during the early preparalytic stage of the disease.

Essential confirmation of the results obtained with the SK and RMV strains of monkey virus can be found in the data given in Table VI which deal with the experiments in which poliomyelitic infection was produced by Aycock virus. It will be noted that of a total of 45 monkeys, treated with murine virus between the 1st and the 5th day of the disease, 24 failed to show any paralytic symptoms, whereas all 30 controls developed paralysis, among the 30 paralyzed controls paralysis was partial in only 6 animals, the remaining 24 controls succumbing to the disease with complete prostration. It should be added that among the 21 treated animals which developed paralysis (11 complete and 10 partial), there were 4 monkeys which ran an atypical course of the disease.

TABLE V

Interference between Murine Virus and Monkey Virus (RMV Strain) in Therapeutic Experiments

Experiment No	No of monkeys	Infection with monkey virus Dose	Mode of murine therapy		Result		
			Interval between infection and therapy	Injections of murine virus	Complete paralysis	Partial paralysis	No paralysis
I	3 murine	0.5 cc 1 10	No interval	6	0	0	3
	3 " "	" " "	5th day of disease	7-8	3	0	0
	3 controls	" " "	—	—	3	0	0
II	5 murine	0.5 cc 1 10	No interval	8-9	1	3	1
	6 " "	" " "	3rd day of disease	6-9	5	1	0
	3 controls	" " "	—	—	3	0	0
III	3 murine	0.5 cc 1 200	No interval	9	0	0	3
	3 controls	" " "	—	—	3	0	0
Totals	20 murine 9 controls				9 9	4 0	7 0

inasmuch as paralysis occurred after greatly prolonged incubation periods (16 to 30 days). The conclusion, therefore, seems justified that the administration of murine virus in monkeys infected with the Aycock strain of poliomyelitis virus had produced distinct therapeutic effects, especially when treatment was instituted within the first 48 hours of the incubation period of the experimental disease.

When all observations relating to the treatment of experimental poliomyelitis by murine virus are considered as a whole, the combined figures of three experimental series indicate that among a total of 88 monkeys, which had received murine virus between the 1st and 5th day of the disease, 51 monkeys, or more than half (57 per cent), failed to show any paralytic symptoms, while in a group of 50 untreated controls only 2 monkeys (4 per cent) escaped the disease. By

limiting the statistical analysis to an evaluation only of the efficacy of early treatment, begun on the day of infection with poliomyelitis virus, it becomes apparent that of a total of 40 monkeys thus treated, 26 animals, or almost two-thirds (65 per cent), remained free from paralysis, as compared with one single non paralytic survivor among 47 accompanying control animals. By contrast, when treatment was delayed until 96 hours after poliomyelitic infection, marked protection was obtained only against the weak SK strain, all treated animals infected with highly virulent RMV or Aycock virus succumbing to the disease like controls. While the above data serve as a measure of the inci

TABLE VI
*Interference between Murine Virus and Monkey Virus (Aycock Strain) in
Therapeutic Experiments*

Experiment No	No of monkeys	Infection with monkey virus Dose	Mode of murine therapy		Result		
			Interval between infection and therapy	Injections of murine virus	Complete paralysis	Partial paralysis	No paralysis
I	1 murine 1 control	0.5 cc. 1:10 " " "	No interval —	1 —	1 (30 days) 0	0 1 (9 days)	0 0
II	1 murine	0.5 cc. 1:20	No interval	1	1 (10 days)	0	0
	3 " "	" " "	" " "	6	0	0	3
	3 " "	" " "	3rd day of disease	7-8	1 (7 days)	2 (10 days)	0
	3 controls	" " "	5th " "	6-7	3 (8 6-7 ")	0 1 (12 days)	0 0
III	6 murine 3 controls	0.5 cc. 1:20 " " "	No interval —	3-6 —	0 3 (7 days)	1 (7 days)	5 0
IV	4 murine	0.5 cc. 1:20	No interval	7-9	2 (9 days)	1 (10 days)	1
	4 " "	" " "	3rd day of disease	7-8	0	1 (12 ")	3
	5 controls	" " "	—	—	4 (5-6 days)	1 (12 ")	0
V	5 murine	0.5 cc. 1:50	No interval	6	2 (7-8 days)	1 (16 days)	2
	5 " "	" " "	3rd day of disease	6-8	0	2 (8-9 ")	3
	5 controls	" " "	—	—	3 (7-8 days)	2 (9 ")	0
VI	6 murine	0.5 cc. 1:50	No interval	9	0	1 (11 days)	5
	4 " "	" " "	3rd day of disease	9	1 (20 days)	1 (20 ")	2
	11 controls	" " "	—	—	10 (6-13 ")	1 (13 ")	0
Totals	45 murine 30 controls				11 24	10 6	24 0

dence of the disease in the treated and the control group, the effects of treatment with murine virus can also be gauged by comparing the severity of the disease in the two groups of animals. Such a comparison, whether applied to the total figures or to figures relating to early treatment, reveals that the percentage of prostrating paralysis in treated animals was always a fraction of that occurring in untreated control animals, i.e., 22 per cent against 72 per cent for the total group and 17 per cent against 82 per cent for the early treatment group. That early treatment with murine virus, when successful, virtually aborts the experimental disease, whereas delayed treatment, even though saving the animal from paralysis is unable to prevent some form of subclinical infection is also

strikingly demonstrated by the results of reinfection experiments. Thus, in a group of 6 surviving monkeys, in which treatment had been begun on the 1st day of infection, none escaped paralysis upon reinfection with Aycock or RMV virus, by contrast, in another group of 9 surviving monkeys, which had been treated at the 48 or 96 hour interval, 8 proved resistant and only 1 susceptible to reinfection with the same viruses.

Murine virus being capable of bringing about as powerful protective effects as would appear from these data, it may be pertinent to raise the question why it has not been possible to abort the disease in an even higher percentage of monkeys during the early stages of the incubation period. This question cannot be readily answered. As far as the fate of murine virus is concerned, it apparently persists for some time, in active form, in the central nervous system of monkeys when introduced shortly after poliomyelitic infection. For on three occasions, when monkeys injected with murine virus on the day of infection with poliomyelitis virus were sacrificed between the 1st and 7th day during the incubation period, transfers of brain and cord to mice revealed the presence of large amounts of murine virus. On the other hand, when monkeys developed poliomyelitis despite treatment with murine virus, it has usually been impossible to demonstrate any murine virus in the central nervous system of such paralyzed animals. Thus, transfers to mice of brain and cord from 4 prostrate treated monkeys, carried out at intervals of from 1 to 3 days after the last injection of murine virus, gave no evidence of the existence of any active murine virus in these tissues. All that can be said, therefore, is that the failure of interference seems to be associated with the absence of murine virus, while the data are not inconsistent with the assumption that successful interference depends upon the persistence of murine virus in active form. The lack of success in therapeutic experiments is therefore probably conditioned, partly by the existence of a proper balance between monkey and murine virus—as determined by the initial quantities of virus injected and the rate of their subsequent propagation—and partly by the maintenance of a definite threshold level of murine virus throughout the preparalytic stage of the disease. The harmonious coordination of these variables may well be materially affected by certain individual variations in the response of any given monkey to the two viruses.

DISCUSSION

The data presented in this paper show that the murine strain of SK poliomyelitis virus is capable of interfering with the development of poliomyelitic infection in *rhesus* monkeys. Murine infection in mice, on the other hand, is not significantly influenced by the administration of monkey poliomyelitis virus, nor was the growth of murine virus inhibited in tissue cultures to which monkey poliomyelitis virus had been added. This interference phenomenon, therefore, appears to be a unilateral reaction in that the stronger murine virus dominates over the weaker, simian strains. Such interference, as can be

demonstrated in monkeys, operates effectively not only against the parent SK monkey strain but also against two other highly virulent strains of monkey passage virus, *i.e.* Aycock and RMV. It is further evident that interference takes place, irrespective of whether monkey and murine virus are injected in form of *in vitro* prepared mixtures, or whether the two viruses are introduced by separate routes. When murine virus is given intravenously to monkeys before or after intracerebral infection with monkey virus, distinct prophylactic and therapeutic effects may be obtained. The limits of effective interference are set by certain critical thresholds of time and dosage which seem to govern the interaction between the two opposing viruses. Thus, the weaker culture virus makes a less effective interfering agent than highly potent mouse passage virus (15), whereasavian passage virus, which possesses even lower virulence, has given no clear-cut evidence of therapeutically effective interference (17). It may be added that no protection occurs when intravenous injections of monkey virus are substituted for murine virus during the incubation period of the experimental disease.

The protection which is induced when murine virus interferes with the development of poliomyelitic infection in monkeys is probably not referable to any immunizing effects, humoral or cellular, of the murine strain. Thus, previous experience has demonstrated that prolonged immunization of monkeys with poliomyelitis virus, be it of simian or of murine origin, causes but rarely a state of resistance sufficiently marked to protect the immunized animal against intracerebral infection with monkey passage virus. Furthermore, protection in interference experiments is afforded in prophylactic as well as in therapeutic tests. It must also be remembered that monkeys which have survived the experience of *in vivo* interference between murine and monkey virus, as a rule, remain fully susceptible to subsequent reinfection with monkey virus. All these observations point in the direction of an immediate, though transient reaction which differs, both in its speed and lack of persistence, from classical immunological processes.

While it seems permissible to exclude immunity as being responsible for the observed protection, no explanation which pretends to have more than heuristic significance can be offered at this time for the mechanism of interference. Rivers (18) has drawn attention to a general impression, prevailing among virus workers, that unhealthy animals are either more resistant or react less severely to certain virus maladies than do perfectly healthy animals. Proceeding from this experience to a discussion of the known systems of viral interference he has suggested that normal cells might be more suitable for the multiplication of a virus than cells rendered abnormal by previous contact with another virus. This, of course, is merely a suggestion and it becomes necessary to subject the available data to a critical analysis if we expect to reach a better understanding of these obscure phenomena.

To begin with, it seems fairly obvious that the various manifestations of viral

antagonism, which, for want of a better term, have been loosely brought together under the name of "interference," make up a rather heterogeneous group in so far as their *modus operandi* is concerned. Such sparing effects, for instance, as are demonstrable between poliomyelitic and lymphocytic choriomeningitic infection, have probably no connection whatsoever with intrinsic properties of the inciting agents, since the two diseases are caused by totally unrelated viruses. The simplest explanation of the phenomenon would be to assume that poliomyelitis virus, on its way from brain to cord, is partially or completely intercepted by the barrier of an extensive inflammatory reaction which constitutes the most characteristic feature of lymphocytic choriomeningitic lesions. In other words, the failure of poliomyelitis virus to produce paralysis, in this instance, is probably due to an essentially mechanical restriction of virus to its primary site of inoculation. On the other hand, different conditions seem to obtain in those cases of viral interference in which the competing viruses represent pathogenic and non-pathogenic variants of the same strain, or are otherwise closely related. We are referring to such interference as occurs between the neurotropic and viscerotropic descendants of yellow fever virus or between the virus of yellow fever and the virus of Rift Valley fever, for even though the last two viruses and the diseases which they produce are seemingly unrelated, sufficient analogies exist to raise the question whether both viruses may not have originated from some common ancestral form (10). Obviously, the interference that is demonstrable between the pantropic murine strain of SK poliomyelitis virus and the neurotropic simian strains of poliomyelitis virus falls into the same category, and we probably do not go far astray by assuming that the basic mechanisms responsible for the several interference phenomena listed in this group are very similar. Certainly, all three types of interference just mentioned operate with singular efficiency since protection can be obtained with great regularity against multiple infecting doses of highly virulent virus. As far as information is concerned that has come to us from a study of the poliomyelitis interference system, the available data suggest that the success of interference is associated with the survival, and failure with the absence, of murine virus in the central nervous system of the monkey. It may further be taken for granted that a definite correlation exists between the potency level of murine virus, as determined by titration in mice, and its interfering ability, as tested in monkeys. Protection, therefore, seems to result from domination of one virus over the other. Precisely what the mechanism of this domination is, is impossible to say at present. It may either be mediated, in some way, by a reaction on the part of mutually susceptible cells, or else be brought about by direct virucidal interaction between the two viruses themselves. The first hypothesis seems the more plausible since both viruses, though differentially pathogenic for monkeys and mice, possess the same affinity for the anterior horn cell which constitutes the selec-

tive seat of the poliomyelitic lesion. Such a "blockade" of susceptible cells by non paralyzing murine virus might render these cells temporarily impregnable to an attack of paralyzing monkey virus because the orderly function of certain enzyme systems, necessary for successful propagation of monkey virus, has conceivably been disturbed by previous contact with murine virus. However, it should be mentioned that positive interference has also been obtained with murine virus preparations which had been partially inactivated by exposure to ultraviolet light. Such irradiated virus, innocuous for mice by intraperitoneal injection though still mildly infectious by intracerebral test, has proven an effective interfering agent, on several occasions in both therapeutic and mixture experiments. The question arises, therefore, whether the interfering principle in murine virus is identical with the infectious unit itself, or whether interference is brought about by a non-infectious substance, existing as an integral part of this unit or occurring separately in soluble form. It is hoped that further investigations, which are in progress, will help to throw light on this fundamental problem.

SUMMARY AND CONCLUSIONS

1 The murine strain of SK poliomyelitis virus interferes with the propagation in *rhesus* monkeys of SK, Aycock, and RMV poliomyelitis monkey virus.

2 This interference is demonstrable by intracerebral injection of mixtures of murine and monkey virus prepared *in vitro* as well as by separate injection of the two viruses by diverse routes.

3 Mixture tests carried out with graded doses of murine and monkey virus show that 0.5 cc. of a 10 per cent suspension prepared from the brains of paralyzed mice is capable of counteracting at least 100 minimal paralyzing doses of two strains of monkey virus.

4 No interference was demonstrable with suspensions of brains infected with murine virus which had been inactivated by heating for $\frac{1}{2}$ hour at 75°C, or with suspensions prepared from normal mice, or with brain suspensions prepared from mice infected with herpes virus.

5 When murine virus is introduced into monkeys by the intravenous route, before or after intracerebral infection with monkey virus, distinct prophylactic or therapeutic results may be obtained.

6 Analysis of the figures shows that the success of interference depends upon (a) the size of the infecting dose of monkey virus, (b) the amount of murine virus injected, and (c) the choice of proper intervals between the injection of monkey and murine virus.

7 The mechanism of the interference phenomenon here described is discussed in the light of the available data.

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HEPATIC VITAMIN A IN THE RAT AS AFFECTED BY THE ADMINISTRATION OF DIBENZANTHRACENE*

BY JULES C. ABELS,† M.D. ALICE T. GORHAM SHIRLEY L. EBERLIN
ROBERT HALTER, PH.D. AND C. P. RHOADS, M.D.

(From the Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York)

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Considerable experimental evidence exists to prove that the administration of the carcinogen dibenzanthracene to the rat results in a decrease of the content of vitamin A in the animal's liver (1, 2). This result is obtained when the animal receives either adequate or moderately excessive amounts of the vitamin (2), but the mechanism by which the effect is produced is entirely unknown. Recently, however, the possibility has been suggested (3) that a specific hepatic protein, to which vitamin A conceivably is bound, is in some manner impaired or absent from the liver of the animal which receives the carcinogen.

This suggestion of damage to a conjugated protein, of which vitamin A forms the prosthetic group, may be analogous to the concept of Kensler, Dexter, Young, and Rhoads (4, 5). They demonstrated that the presence of metabolites of the carcinogen butter yellow prevents *in vitro* both the diphosphopyridine nucleotide and cocarboxylase from functioning with their specific protein enzymes in the yeasts used. This prevention, in turn, can be avoided, within limits, by the introduction of excessive amounts of the specific coenzymes into the systems. It is conceivable, therefore, that a similar competitive relationship might exist in the rat liver between vitamin A and dibenzanthracene—a competition for a specific protein, perhaps of enzyme nature, and the success in that competition dependent upon the relative concentrations of the two competing substances.

On the other hand, since one of the recognized functions of the liver is to fabricate, store, and distribute vitamin A (6), the impaired hepatic storage of the vitamin due to dibenzanthracene feeding also might be explained by the production of a general hepatic insufficiency imposed on the organ by the carcinogen. The association of a defective distribution of vitamin A between the liver and plasma of patients with gastro-intestinal cancer (7) with the presence of a generalized hepatic damage in those patients (8) already has been demonstrated. Likewise, there is good evidence that some patients with hepatic cirrhosis or atrophy have lost, to a considerable extent, their ability to store the vitamin (9).

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† Finney Howell Fellow

In view of these facts, it became desirable to investigate the evidence in support of these two possible explanations for the defective storage of the vitamin in the dibenzanthracene treated rat (1) that the carcinogen has a specific effect on some liver protein, or other anchor, to which the vitamin normally is attached, or (2) that the carcinogen causes a general hepatic insufficiency and among the functions lost is that of vitamin A storage. The results of this investigation form the subject of the present report.

Methods and Materials

The experiments were carried out on male albino rats of Sherman strain, which had an initial weight of from 90 to 110 gm.

The standard diet consisted of 8 to 9 gm of Purina dog chow (Ralston Purina Company) per rat, per day¹. To this daily ration were added 1 gm of carrot and 1.5 gm of lettuce. In certain experiments 1.5 gm of Fleischmann's dried brewer's yeast, No. 20-40,² were added to the daily diet of each rat.

Vitamin A was given in the form of a vitamin A concentrate³ diluted in corn oil so that 1 ml contained 400 U.S.P. units of the vitamin. The required amount of this supplement was administered daily through a syringe inserted into the mouths of those animals which received the vitamin.

A 0.1 per cent emulsion of dibenzanthracene (10) was injected in doses of 3 mg or 5 mg at weekly intervals into the peritoneal cavity of those rats scheduled to receive the carcinogen. The total volume of each dose varied from 3 to 5 ml.

No animal was given either vitamin A supplement or dibenzanthracene until he had received the standard diet for 2 weeks in order to make sure that all the rats were in good condition and to allow them to become adjusted to their environment. Urine collections were obtained from each group of animals for 5 days before they were sacrificed. During this collection period, the animals to be killed were kept in metabolism cages.

All animals were weighed every week. In general, the nutrition of the control animals was found to be well maintained and their weight increased uniformly at about 15 gm per rat, per week for 12 weeks.

Animals were sacrificed by decapitation to procure adequate amounts of blood. As soon as possible, the liver of each rat was removed and weighed. A portion of liver of about 600 mg then was placed in a sealed, tared weighing bottle. This specimen was weighed accurately and homogenized into a brei for chemical determinations.

In the present study chemical determinations were made of

1. Vitamin A in the plasma and liver. The methods used have been described previously by the authors (7, 11).

¹ This ration contained from 150 to 170 U.S.P. units of vitamin A.

² The yeast was supplied through the courtesy of the Standard Brands, Inc. 1 gm of yeast contained 520 mg of protein, 160 μ g of thiamin, 70 μ g of riboflavin, 60 μ g of pyridoxin, 600 μ g of nicotinic acid, and about 650 μ g of choline. It is also known to contain appreciable amounts of sulfur amino acids.

³ This concentrate was supplied through the courtesy of the Endo Products, Inc.

2. Total protein, albumin, and globulin of the serum and liver⁴ The technique of Robinson *et al* (12) was used to measure the protein and its fractions in the serum and liver brei

3 The total lipid carbon of the liver This was determined by the technique of Van Slyke *et al* (13)

4 The cholesterol and cholesterol esters of the serum. These were measured by the method of Schoenheimer and Sperry (14)

5 Phospholipids of the liver The method of Sinclair (15) was used.

6 Riboflavin of the liver The technique of Hodson and Norris (16) adapted by Kensler *et al* (4) for the determination of the vitamin in liver brei was employed

7 The urinary glucuronates. These were measured by the procedure of Maughan *et al* (17)

8 The urinary phenol and phenol esters. The method of Folin and Denis was used (18) The final color developed was read by the Pfalz and Bauer Photoelectric colorimeter

The material now to be presented includes the results of experiments to determine whether the administration of dibenzanthracene to the rat resulted in (a) a general hepatic insufficiency, or (b) an isolated effect on a specific liver protein (or other anchor) to which vitamin A normally is attached

Experiments to Ascertain Whether or Not the Administration of Dibenzanthracene Produces a General Hepatic Insufficiency

For these experiments a colony of 58 Sherman strain, male albino rats was used These animals had an initial weight of from 90 to 110 gm After they had taken the standard diet for 2 weeks, 10 unselected rats were sacrificed in order to ascertain the status of the hepatic functions of the animals in the colony

Results of Preliminary Studies—At the time the 10 animals were sacrificed their weights ranged from 126 to 140 gm. and averaged 134.5 gm. The livers of these animals weighed from 4.0 to 6.1 gm., and the average weight was 4.9 gm. Thus, the ratio of liver weight to body weight ranged from 0.035 to 0.039, and the average ratio was 0.037 (Table I)

The criteria used in this study as measures of normal hepatic function were the ability of the liver to (1) maintain a normal total lipid and phospholipid content, (2) to store and fabricate albumin, (3) to esterify cholesterol, (4) to synthesize and conjugate glucuronic acid, and (5) to conjugate phenol The justification for the use of these criteria is presented in a later section of this communication

Accordingly, determinations were made in these and all other animals of this colony which subsequently were sacrificed, of the hepatic concentrations of total

⁴ By the term liver albumin is meant that fraction of the liver protein soluble in 22 per cent Na_2SO_4 at 37°C. by the term liver 'globulin' is meant the total liver protein less the liver 'albumin.'

(c) *The Concentrations of Vitamin A in the Liver* The effect of the administration of dibenzanthracene on the hepatic store of vitamin A is essentially that obtained by Goerner (1) and by Baumann (2, 3) Even at the end of 4 weeks, when each of the rats of groups C and D had received only 12 mg of the car-

TABLE II

The Average Body Weight of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of		
	4 wks	8 wks	12 wks
	gm	gm	gm
A	210	265	270
B	213	250	245
C	181	213	170
D	179	218	210

Group A received basal diet alone, group B received diet and 200 u.s.p. units of vitamin A per rat, per day, group C received diet and 3 mg of dibenzanthracene per rat, per week, group D received diet and 200 u.s.p. units of vitamin A per rat, per day, and 3 mg of dibenzanthracene per rat, per week.

TABLE III

The Average Liver Weight and Ratios of Liver Weights to Body Weights of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of					
	4 wks		8 wks		12 wks	
	Liver weight	LW/BW	Liver weight	LW/BW	Liver weight	LW/BW
	gm		gm		gm	
A	7.4	0.035	8.6	0.032	6.05	0.022
B	7.6	0.036	6.3	0.025	6.8	0.028
C	9.6	0.052	9.75	0.040	7.9	0.046
D	9.3	0.050	11.2	0.041	9.5	0.045

Group A received basal diet alone, group B received diet and 200 u.s.p. units of vitamin A per rat, per day, group C received diet and 3 mg of dibenzanthracene per rat, per week, group D received diet and 200 u.s.p. units of vitamin A per rat, per day, and 3 mg of dibenzanthracene per rat, per week.

cinogen, significant decreases in their hepatic concentrations of vitamin A were noted. The average hepatic content of the vitamin of group A was 266 u.s.p. units, of group B, (which had received 200 u.s.p. units of vitamin A per rat per day) 404 u.s.p. units, of group C, 104 u.s.p. units, and of group D (which had received both carcinogen and 200 u.s.p. units of vitamin A per rat, per day), 190 u.s.p. units per gm of wet liver (Table IV).

A consistent increase in the average hepatic concentration of vitamin A in all instances was associated with the increased age and weight of the animals. Nevertheless, the hindrance to vitamin A storage which the dibenzanthracene imposed upon the livers was a constant finding. At the end of 8 weeks, the average hepatic concentrations of the animals sacrificed from groups A, B, C, and D respectively were 1192, 1788, 479, and 306 u.s.p. units per gm., and those sacrificed at the end of 12 weeks, 2560, 2475, 645, and 554 u.s.p. units per gm.

At the end of the 4 and 8 week periods, the daily administration of 200 u.s.p. units of vitamin A to the rats of group B had increased the hepatic concentra-

TABLE IV

The Average Concentration of Vitamin A in the Livers of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of		
	4 wks.	8 wks.	12 wks.
	U.S.P. units per gm.	U.S.P. units per gm.	U.S.P. units per gm.
A	266	1192	2560
B	404	1788	2475
C	104	479	645
D	190	306	554

Group A received basal diet alone; group B received diet and 200 u.s.p. units of vitamin A per rat, per day; group C received diet and 3 mg. of dibenzanthracene per rat, per week; group D received diet and 200 u.s.p. units of vitamin A per rat, per day and 3 mg. of dibenzanthracene per rat, per week.

TABLE V

The Average Concentration of Vitamin A in the Plasma of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of		
	4 wks.	8 wks.	12 wks.
	U.S.P. units per 100 ml.	U.S.P. units per 100 ml.	U.S.P. units per 100 ml.
A	125	95	200
B	155	160	175
C	202	77.5	150
D	219	143.5	310

Group A received basal diet alone; group B received diet and 200 u.s.p. units of vitamin A per rat, per day; group C received diet and 3 mg. of dibenzanthracene per rat, per week; group D received diet and 200 u.s.p. units of vitamin A per rat, per day and 3 mg. of dibenzanthracene per rat, per week.

tions of the vitamin over those of group A. In contrast to this finding, it was noted that the administration of an equal amount of vitamin A to the rats of group D increased the hepatic levels of the vitamin over those of group C only until the end of the 4th week, and that thereafter, with the continued injections of dibenzanthracene, no increased stores of the vitamin could be effected in the rats of group D.

In summary, therefore, it would appear that the ingestion of vitamin A supplements temporarily increased the hepatic store of the vitamin, and that the parenteral administration of the carcinogen hindered the establishment of this store both in animals taking a normal diet and in those fed 200 u.s.p. units of vitamin A.

(d) *The Concentrations of Vitamin A in the Plasma* At the end of the 4th week the average plasma levels of vitamin A in groups A, B, C, and D in that order were 125, 155, 202, and 219 U.S.P. units per cent. The ingestion of vitamin A supplements was reflected in its increased plasma levels of groups B and D, over those of groups A and C respectively (Table V).

The average concentration of the vitamin in the plasma of the group D rats remained higher than that of the group C animals throughout the experiment, probably due to the continued administration of the vitamin supplements. This relationship, however, did not persist between the animals of groups A and B whose liver concentrations of vitamin A had increased considerably as the experiment continued. A lack of correlation between the plasma levels of vitamin A in rats with normal and abnormally high hepatic concentrations of the vitamin has been found by others (19).

It is of interest to note that after the 4th week the levels of vitamin A in the plasma of groups C and D were no longer correspondingly higher than those of groups A and B.

(e) *Total Lipid Carbon and Phospholipid Content of the Liver* The concentrations of total lipid carbon in the livers of the animals which received the carcinogen were not abnormally increased at the end of the 4th week. The average hepatic contents of total lipid carbon of groups A and B were 5.5 and 9.8 gm per cent respectively, whereas those of groups C and D were 6.4 and 6.8 gm per cent respectively. The average concentrations obtained for the livers of groups A, B, C, and D in that order at the end of 8 weeks were 8.8, 9.3, 10.6, and 7.4 gm per cent, and at the end of 12 weeks 6.5, 5.5, 7.5, and 6.6 gm per cent (Table VI).

This failure to find any significantly altered total lipid carbon content in the livers of the rats which received dibenzanthracene is compatible with the relatively constant concentration of phospholipid in those organs. For groups A, B, C, and D, respectively, the hepatic concentrations of phospholipid were at the end of 4 weeks 2.7, 2.7, 2.85, and 2.25 gm per cent, at the end of 8 weeks 3.0, 2.8, 3.1, and 2.95 gm per cent, and at the end of 12 weeks 2.65, 2.8, 2.9, and 2.75 gm per cent (Table VI).

(f) *The Concentrations of Total Protein, "Albumin," and "Globulin" in the Liver* No consistently significant changes in the concentrations of hepatic protein or the two fractions were noted in the groups of animals studied. At the end of the 4th week, the concentrations of liver total proteins were for groups A, B, C, and D 15.4, 14.6, 13.95, and 14.65 gm per cent respectively. The low value of 13.9 gm per cent of group C was due to a decrease in the "globulin" fraction. The average concentrations of "globulin" at this time were for groups A, B, C, and D respectively 13.7, 12.9, 12.1, and 12.9 gm per cent, and those of "albumin" 1.7, 1.7, 1.85, and 1.75 gm per cent (Table VII).

Four and 8 weeks later the average contents of total protein, "albumin," and

"globulin" in the livers of the group C rats were found to be within the limits of normal. The average values found for total liver protein at the end of 8

TABLE VI

The Average Concentrations of Total Lipid Carbon and of Phospholipid in the Livers of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of					
	4 wks.		8 wks.		12 wks.	
	Total lipid carbon	Phospholipid	Total lipid carbon	Phospholipid	Total lipid carbon	Phospholipid
	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent
A	5.5	2.7	8.8	3.0	6.5	2.65
B	9.8	2.7	9.3	2.8	5.5	2.8
C	6.4	2.85	10.6	3.1	7.5	2.9
D	6.8	2.25	7.4	2.95	6.6	2.75

Group A received basal diet alone; group B received diet and 200 u.s.p. units of vitamin A per rat per day; group C received diet and 3 mg. of dibenzanthracene per rat, per week; group D received diet and 200 u.s.p. units of vitamin A per rat per day and 3 mg. of dibenzanthracene per rat, per week.

TABLE VII

The Average Concentrations of Total Protein, Albumin, and Globulin in the Livers of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of								
	4 wks.			8 wks.			12 wks.		
	Total protein	Albumin	"Globulin"	Total protein	"Albumin"	"Globulin"	Total protein	Albumin	Globulin
	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent
A	15.4	1.7	13.7	16.75	2.55	14.2	19.0	4.1	14.9
B	14.6	1.7	12.9	18.3	2.7	15.6	18.65	3.75	14.9
C	13.95	1.85	12.1	18.45	2.85	15.6	18.7	3.8	14.9
D	14.65	1.75	12.9	14.9	2.1	12.8	19.2	3.6	15.6

Group A received basal diet alone; group B received diet and 200 u.s.p. units of vitamin A per rat, per day; group C received diet and 3 mg. of dibenzanthracene per rat, per week; group D received diet and 200 u.s.p. units of vitamin A per rat, per day and 3 mg. of dibenzanthracene per rat, per week.

weeks for groups A, B, C, and D in that order were 16.75, 18.3, 18.45, and 14.9 gm. per cent, and at the end of 12 weeks 19.0, 18.65, 18.7 and 19.2 gm. per cent. The average concentrations of "albumin" at the end of 8 weeks were 2.55, 2.7, 2.85, and 2.1 gm. per cent and at the end of 12 weeks 4.1, 3.75, 3.8, and 3.6

gm per cent for groups A, B, C, and D respectively. Finally, at the end of 8 weeks, the average hepatic contents of "globulin" were 14.2, 15.6, 15.6, and 12.8 gm per cent, and at the end of 12 weeks 14.9, 14.9, 14.9, and 15.6 gm per cent for groups A, B, C, and D respectively.

(g) *The Concentrations of Total Protein, Albumin, and Globulin in the Serum*
Likewise, no evidence of a significant hypoproteinemia or hypoalbuminemia was found in the animals which were injected with dibenzanthracene. During the 12 weeks of this experiment, the average total serum protein of group A varied from 6.2 to 7.2 gm per cent, of group B from 6.0 to 7.1 gm per cent, of group C from 6.1 to 6.9 gm per cent, of group D from 6.0 to 7.3 gm per cent.

TABLE VIII

The Average Concentrations of Total Protein, Albumin, and Globulin in the Serum of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of								
	4 wks			8 wks.			12 wks		
	Total protein	Albumin	Globulin	Total protein	Albumin	Globulin	Total protein	Albumin	Globulin
	gm per cent	gm per cent	gm per cent	gm per cent	gm per cent	gm per cent	gm per cent	gm per cent	gm per cent
A	7.2	4.0	3.2	6.2	3.9	2.3	6.8	4.9	1.9
B	7.1	4.4	2.7	6.0	3.6	2.4	6.4	5.4	1.0
C	6.9	3.9	3.0	6.1	3.6	2.5	6.4	5.5	0.9
D	7.3	4.3	4.0	6.0	3.4	2.6	6.75	5.7	1.05

Group A received basal diet alone, group B received diet and 200 U.S.P. units of vitamin A per rat, per day, group C received diet and 3 mg of dibenzanthracene per rat, per week, group D received diet and 200 U.S.P. units of vitamin A per rat, per day, and 3 mg of dibenzanthracene per rat, per week.

The average concentration of serum albumin of group A varied from 3.9 to 4.9 gm per cent, of group B from 3.6 to 5.4 gm per cent, of group C from 3.6 to 5.5 gm per cent, and of group D from 3.4 to 5.7 gm per cent (Table VIII).

Furthermore, the average ratios of albumin to globulin in the serum of groups C and D were not significantly lower than those of groups A and B. These values for groups A, B, C, and D in that order were at the end of 4 weeks 1.25, 1.63, 1.30, and 1.43, at the end of 8 weeks, 1.70, 1.50, 1.44, and 1.31, and finally at the end of 12 weeks, 2.46, 5.40, 6.34, and 5.43. The concentrations of globulin in the serum of the four groups varied considerably and accordingly these variations were reflected in the wide fluctuation of the A/G ratios.

(h) *The Ratios of the Concentration of Esterified to Total Cholesterol in the Serum*
At no time were the ratios of esterified to total cholesterol abnormally altered in the serum of those rats which received the carcinogen. After the

animals of groups C and D had each received the dibenzanthracene for 4 weeks, these ratios were 70 and 75 per cent respectively, whereas those for groups A and B were 75 and 70 per cent respectively. Again, at the end of 8 weeks, the ratios for groups A, B, C, and D in that order were 86, 84, 71, and 85 per cent, and at the end of 12 weeks 73, 62, 66, and 72 per cent (Table IX)

(i) *The Daily Urinary Excretion of Glucuronates, and of Total and Conjugated Phenols* The urine was collected from each group of animals for 5 days during the 4th, 8th, and 12th weeks of the experiment. Determinations of the daily output of glucuronates, however, were made only during the 4th and 12th weeks, and at those times no significantly abnormal decrease in the amounts excreted by groups C and D were found. During the 4th week, the animals

TABLE IX

The Average Concentrations of Free and Esterified Cholesterol in the Serum of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of					
	4 wks		8 wks.		12 wks.	
	Free	Esters	Free	Esters	Free	Esters
	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
A	30	77.5	11	68	23	60
B	34	79	13	68	33	53
C	22	103	20	50	31	61
D	30	92	13	75	24	63

Group A received basal diet alone; group B received diet and 200 U.S.P. units of vitamin A per rat, per day; group C received diet and 3 mg of dibenzanthracene per rat, per week; group D received diet and 200 U.S.P. units of vitamin A per rat, per day and 3 mg of dibenzanthracene per rat, per week.

of groups A, B, and C excreted 4.5 mg per rat per day, and those of group D 5.0 mg per rat per day. During the 12th week, group A excreted 4.0 mg per rat per day, group B 5.5 mg, group C 5.0 mg, and group D 4.25 mg per rat, per day.

During the periods of collection the daily excretion of total and of conjugated phenol by the animals in groups C and D were within the limits of those excreted by the rats which did not receive the carcinogen. The urinary output of total phenols by groups A, B, C, and D respectively were 2.8, 4.0, 3.2, and 3.3 mg per day during the 4th week, 4.1, 1.9, 4.7, and 3.55 mg per day during the 8th weeks, and 3.8, 3.1, 4.2 and 3.8 mg per day during the 12th week. Furthermore, the degree of phenol conjugation by the rats of groups C and D during these periods were at all times within the normal limits. The ratio of the outputs of total to conjugated phenol of groups A, B, C, and D in that

order were 23, 26, 30, and 24 per cent in the 4th week, 33, 22, 23, and 24 per cent in the 8th week, and 34, 34, 37, and 37 per cent in the 12th week (Table X)

(g) *The Concentration of Riboflavin in the Liver* Goerner has demonstrated

TABLE X

The Average Excretion of Glucuronates, Total Phenol, and Phenol Esters in the Urine of the Rats in the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	During the course of the							
	4th wk			8th wk		12th wk		
	Glucuro nates	Total phenol	Phenol esters	Total phenol	Phenol esters	Glucuro nates	Total phenol	Phenol esters
	mg /rat per day	mg /rat per day	mg /rat per day	mg /rat per day	mg /rat per day	mg /rat per day	mg /rat per day	mg /rat per day
A	4.5	2.8	0.6	4.1	1.7	4.0	3.8	1.3
B	4.5	4.0	1.05	1.9	0.4	5.5	3.1	1.05
C	4.5	3.2	1.0	4.7	1.1	5.0	4.2	1.6
D	5.0	3.3	0.8	3.55	0.85	4.25	3.8	1.4

Group A received basal diet alone, group B received diet and 200 u.s.p. units of vitamin A per rat, per day, group C received diet and 3 mg of dibenzanthracene per rat, per week, group D received diet and 200 u.s.p. units of vitamin A per rat, per day, and 3 mg of dibenzanthracene per rat, per week.

TABLE XI

The Average Concentration of Riboflavin in the Livers of the Rats on the Basal Diet and of Those Which Received Supplements of Vitamin A and of Dibenzanthracene

Group	At the end of		
	4 wks	8 wks	12 wks
	μg	μg	μg
A	17	30	34
B	17	33	30
C	15	30.5	28
D	15	29	30

Group A received basal diet alone, group B received diet and 200 u.s.p. units of vitamin A per rat, per day, group C received diet and 3 mg of dibenzanthracene per rat, per week, group D received diet and 200 u.s.p. units of vitamin A per rat, per day, and 3 mg of dibenzanthracene per rat, per week.

that whereas the administration of dibenzanthracene to the rat depletes and prevents the establishment of the hepatic store of vitamin A, the carcinogen is without effect upon the concentrations of vitamin C in the liver (1). To investigate further the possibility that the primary effect of the dibenzanthracene is on the hepatic store of vitamin A, determinations were made of the concentrations of riboflavin in the livers of the animals studied.

At the end of the 4th week of the experiment, the hepatic concentrations of riboflavin were for groups A, B, C, and D respectively 17, 17, 15, and 15 μg per gm wet weight. At the end of the 8th week, the average concentrations were 30, 33, 30.5, and 29 μg per gm., and at the end of the 12th week, 34, 30, 28, and 30 μg per gm wet weight for groups A, B, C, and D respectively (Table VI)

Thus, it would appear that the administration of the carcinogen depletes neither the hepatic store of vitamin C nor that of vitamin B₂.

The evidence at hand indicates, therefore, that the parenteral administration of dibenzanthracene to rats does produce an hepatomegaly but not a general hepatic insufficiency. This conclusion is based upon the observation that the rats which received the carcinogen did not have any significant fatty infiltration of their livers nor abnormally low hepatic content of phospholipids. The livers contain normal amounts of "albumin" and make normal amounts of serum albumin, esterify normal amounts of cholesterol and phenol, and synthesize and conjugate normal amounts of glucuronic acid. Furthermore, the administration of the carcinogen to the rats does not impair the ability of their livers to store all vitamins, since such livers retain normal concentrations of riboflavin and of vitamin C (1).

Experiments to Ascertain Whether or Not a Competition Exists Between Dibenzanthracene and Vitamin A for the Storage of the Vitamin in the Liver

From the data presented it would appear that the injection of dibenzanthracene into the rat in some manner impairs the ability of vitamin A to remain in the liver. A similar situation has been found to exist when the carcinogen, butter yellow, is fed to the rat (4). The administration of this carcinogen decreases the hepatic stores of diphosphopyridine nucleotide and of riboflavin. Furthermore, the introduction of certain metabolites of butter yellow into a suspension of yeast apozymase has been shown to reduce the activity of diphosphopyridine nucleotide, a derivative of nicotinic acid, probably by blocking the nucleotide from its attachment to its specific enzyme (20). This block, however, can be prevented *in vitro* by the introduction into the system of excess amounts of the nucleotide. This fact suggests the existence of a competition *in vitro* between the metabolite of butter yellow and the diphosphopyridine nucleotide for the specific enzyme protein, and perhaps a similar competition exists in the liver of the animal fed butter yellow. Hence should it be possible to demonstrate that the ability of dibenzanthracene to reduce the liver content of vitamin A could be prevented by the simultaneous administration of excess amounts of vitamin A, then it would be reasonable to assume the existence of a similar competition between dibenzanthracene and vitamin A for the storage of the vitamin. Furthermore, it would appear

that the presence of dibenzanthracene, or its metabolites, specifically impairs the means by which vitamin A is anchored in the liver

Accordingly, this hypothesis was subjected to a simple experiment. Four groups of Sherman strain rats, E, F, G, and H, whose initial weights varied from 85 to 105 gm were placed on the same diet as that given the animals in the previous experiment. Each rat received at weekly intervals 3 mg of dibenzanthracene intraperitoneally for 8 weeks. Group E consisted of 21 animals which received only the diet and the carcinogen. Group F, of 10 animals, received in addition an oral supplement of 200 U S P units of vitamin A per rat, per day. Group G, of 11 animals, received an oral supplement of 400 U S P units of vitamin A per rat, per day. Finally, the 11 animals of Group H received no supplements of vitamin A, but instead 1.5 gm of Fleischmann's brewers' yeast each day. This last group was included in order to determine whether or not the administration of a crude supplement of the B vitamins and choline also might not counteract the effect of the carcinogen on the hepatic store of vitamin A.

At the end of the 8th week of this experiment, 5 unselected rats of each group were sacrificed and determinations made of their hepatic and blood levels of vitamin A. The average hepatic concentration of the vitamin of groups E, F, G, and H respectively were 750, 1125, 1560, and 394 U S P units per gm wet weight (Table XII). Thus, the values obtained for groups E, F, and G indicated at once that the simultaneous administration of increasing amounts of vitamin A exerted a graded countereffect on the ability of dibenzanthracene to impair the hepatic storage of this vitamin. Indeed, this countereffect was noted in the previous experiment at the end of the first 4 week period. At that time the average vitamin A in the livers of the rats which had received the carcinogen alone (group C) was 104 U S P units per gm, whereas that of the animals which received both the carcinogen and 200 U S P units of vitamin A daily was 190 U S P units per gm. In the previous experiment, however, this relationship no longer existed at the end of the 8th or 12th weeks. Why the daily administration of 200 U S P units of vitamin A in the first experiment counteracted the effect of dibenzanthracene for 4 weeks but not for 8 weeks, whereas it still did so in the second, cannot be explained at this time.

In this second experiment, at the end of 8 weeks the average levels of vitamin A in the plasma of groups E, F, G, and H respectively were 183, 300, 400, and 160 U S P units per cent. For groups E, F, and G these increasing plasma levels probably reflected only the corresponding increase in the hepatic concentration of the vitamin (19) (Table XIII).

It is to be noted that the average concentration of the vitamin in both the liver and plasma of the animals which received yeast (group H) were even lower than those of group E. The animals of the former group had received no less vitamin A than had those of the latter.

Since it was found that the administration of excess amounts of vitamin A could counteract the ability of dibenzanthracene to impair hepatic storage of the vitamin, it was next desirable to determine whether or not the injection of larger amounts of the carcinogen, in turn, could counterbalance the "protective" effects of the vitamin A supplements. Should such a counterbalance be demonstrated then the hypothesis that a competition exists between the carcinogen and vitamin A for the hepatic storage of the vitamin would have some further support.

TABLE XII

The Average Concentration of Vitamin A in the Livers of the Rats Which Received Dibenzanthracene and Supplements of Vitamin A or Yeast

Group	At the end of	
	8 wks.	12 wks.
	U.S.P. units per gm	U.S.P. units per gm
E	750	685
F	1125	664
G	1560	898
H	394	228

Group E received basal diet and 3 mg of dibenzanthracene per rat, per week for 8 weeks and 5 mg of dibenzanthracene per rat, per week thereafter. Group F received, in addition, 200 U.S.P. units of vitamin A per rat, per day; group G received 400 U.S.P. units per rat, per day; group H received 1.5 gm of yeast per rat, per day.

TABLE XIII

The Average Concentration of Vitamin A in the Plasma of the Rats Which Received Dibenzanthracene and Supplements of Vitamin A or Yeast

Group	At the end of	
	8 wks.	12 wks.
	U.S.P. units per 100 ml	U.S.P. units per 100 ml
E	183	288
F	300	300
G	400	250
H	160	275

Group E received basal diet and 3 mg of dibenzanthracene per rat, per week for 8 weeks and 5 mg of dibenzanthracene per rat, per week thereafter. Group F received, in addition, 200 U.S.P. units of vitamin A per rat, per day; group G received 400 U.S.P. units per rat, per day; group H received 1.5 gm of yeast per rat, per day.

In an attempt to provide this evidence the remaining animals of groups E, F, G, and H were given weekly injections of 5, instead of 3, mg of dibenzanthracene. At the end of 12 weeks, after 4 of the larger injections had been administered to each rat, all of the animals were sacrificed and determinations made of vitamin A in their livers and plasma.

Previously it had been demonstrated that at the end of the 8th week the daily ingestion of 200 (group F) and 400 (group G) U.S.P. units of vitamin A by rats which received only 3 mg of dibenzanthracene weekly had increased their average hepatic concentrations of the vitamin by 50 and by 108 per cent respectively over that found in the control group (E). However, after the weekly dose of carcinogen had been raised to 5 mg, the average hepatic concentration of the vitamin had fallen in groups E, F, G, and H respectively to 685, 664, 898,

and 228 U.S.P. units per gm wet weight. Thus, the daily ingestion of 200 U.S.P. units of vitamin A by the rats which received 5 mg of the carcinogen no longer was able to increase the hepatic store of the vitamin, and the daily ingestion of 400 U.S.P. units by these animals was able to effect only an increased hepatic concentration of vitamin A of 31 per cent instead of 108 per cent (Table XII).

Hence, the ability of the dibenzanthracene to interfere with the storage of vitamin A by the liver not only can be counteracted by the simultaneous administration of an excess of vitamin A, but this counteraction, in turn, can be overcome by the addition of larger amounts of the carcinogen. Such a relationship indicates the possible existence of a competition between vitamin A and dibenzanthracene, for some component of the liver, possibly protein, with which vitamin A normally is conjugated. The success in this competition apparently depends upon the relative concentrations of the two competing substances.

At the end of the 12th week, when each rat had received 8 injections of 3 mg of dibenzanthracene, and 4 injections of 5 mg of the carcinogen, the average plasma levels of the vitamin of groups E, F, G, and H were 288, 300, 250, and 275 U.S.P. units. There appears to be no obvious correlation between these levels and those of the livers of the corresponding groups (Table XIII).

It is of interest to note that the average hepatic concentration of vitamin A in the animals which received the supplements of yeast (group H) remained significantly lower than even that of the control group E. Thus, it would appear that the simultaneous administration of yeast to the dibenzanthracene treated rat certainly does not protect the animal from the effects of the carcinogen on the hepatic storage of vitamin A.

DISCUSSION

In the present study an attempt has been made to ascertain whether the ability of dibenzanthracene to impede the storage of vitamin A by the rat liver is due to a general hepatic insufficiency caused by the carcinogen or to an impairment of a specific function of the liver. Inasmuch as the liver performs multiple functions, it is reasonable to believe that no single test could measure adequately the efficiency of the whole organ. Therefore, by the quantitative measurement of various intermediary or end products of liver metabolism, and then by the consideration of the results as a whole, it was felt that the presence or absence of general hepatic damage could be recognized. In this study, measurements of liver function were selected arbitrarily and thus it is necessary to consider the evidence which would indicate that the tests employed in this study provided adequate indices.

1 The Hepatic Concentration of Total Lipid and Phospholipid—It is now a well established fact that the liver is concerned intimately in the intermediary metabolism of lipoids (21) and that in many conditions associated with hepatic

damage there occurs a striking accumulation of fat in the liver cells. An increase in the concentration of neutral fat and a decrease of phospholipids has been found especially in the livers of patients with alcoholic hepatic cirrhosis (22), and in animals given various toxic agents (23).

2 Total Protein, Albumin, and Globulin—The best evidence at hand thus far indicates that the liver is of primary importance in the formation, storage, and exchange of plasma protein. Fibrinogen, and probably the albumins, are produced only by the liver cells (24).

Abnormal levels of circulating plasma protein are reached only when the protein stores, chiefly in the liver, are decreased. The normal animal, depleted of its protein stores, readily can synthesize albumin, globulin and fibrinogen from a proper mixture of amino acids, but those proteins are deposited first in the liver and other organs, and do not appear in the plasma until the body stores have been reestablished. In a damaged liver the synthesis of protein from amino acids is altered, and the circulating concentrations of plasma protein continue to decrease with further hepatic damage. The disappearance of the hepatic stores of protein with damage to the liver now has been shown both experimentally and in human individuals with hepatic cirrhosis (24).

3 Cholesterols and Cholesterol Esters—The serum concentration of total cholesterol and the ratio of free to esterified cholesterol in the normal healthy animal is maintained within rather narrow limits. The esterification of this sterol is accomplished chiefly in the liver and the ability of the damaged liver to perform this function is considerably decreased (25).

4 Glucuronic Acid—It has been demonstrated that several aromatic compounds normally are conjugated with glucuronic acid and excreted as glucuronates in the urine. This conjugation has been considered to be a form of detoxification. The liver is the chief site both for the synthesis of glucuronic acid and its conjugation to other aromatic compounds. Livers of rats injured by phosphorus soon lose their capacity to synthesize glucuronic acid (26).

5 Phenol and Phenol Esters—Detailed studies of the metabolism of phenol have demonstrated that the synthesis of phenol sulfuric and phenol glucuronic acids takes place only in the hepatic parenchyma. The exclusion of the liver from the normal circulation by an Eck fistula eliminates phenol conjugation. Likewise extensive liver injury due to various toxic agents always will lessen phenol conjugation. Extreme and fatal hepatic injury will reduce phenol conjugation to zero (27).

In conclusion, it would appear that adequate measures were taken to demonstrate the possible existence of hepatic insufficiency. The fact that none of the functions studied were altered in the animals indicates that dibenzanthracene is not a general hepatotoxin but, according to the data thus far available, apparently affects particularly that function of the liver which is concerned with the metabolism of vitamin A.

This particular effect of the carcinogen can be obviated to some measure by

CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

VI. STUDIES ON THE NATURE OF THE ENZYMES ASSOCIATED WITH THE PURIFIED VIRUS

By CHARLES L. HOAGLAND, M.D. S. M. WARD, JOSEPH E. SMADEL, M.D.
AND THOMAS M. RIVERS M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, April 21, 1942)

Elementary bodies of vaccinia, obtained by differential centrifugation of the dermal pulp of infected rabbits, have been shown to satisfy many of the criteria laid down by workers in protein chemistry for pure biological entities. Chemical examination of this purified virus has revealed the presence of many substances common to protoplasm, including certain of the vitamin catalysts, and at least one metallic component, copper, which seems to bear an intimate relationship to the final infective product (1-5)

Macfarlane and Salaman (6) have reported the presence of catalase and phosphatase in purified elementary bodies of vaccinia, but were unable to demonstrate dehydrogenase activity of any type, although the reaction of the virus with a variety of dyes and substrates was studied. The assumption by these workers that the catalase and phosphatase activity of their virus preparations was due to the virus, and not to possible tissue contaminants, was based on the observation that phosphatase and catalase were greatly concentrated during the process of virus purification, while dehydrogenases, which were present in high titer in the tissues and cell fragments from which the virus was separated, were absent from the final virus product. The possibility that the occurrence of phosphatase and catalase may have been due to surface adsorption was entertained, but was not considered likely because frequent washing of the virus failed to elute them.

As a continuation of previous studies on the nature of the constituents of purified elementary bodies of vaccinia, we decided to repeat the studies of Macfarlane and Salaman, and to extend them to certain other enzymes which are found in the host tissues from which the virus is prepared, and which might, therefore, be associated with the purified virus. The results of a search for oxidative enzymes have appeared in earlier communications (3, 4)

Materials and Methods

A detailed description of the technique for securing relatively large quantities of purified elementary bodies of vaccinia has been published previously (1). Elementary bodies prepared by this method show a high infectivity elementary body ratio and a constant chemical composition. Although

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hydrolytic enzymes are remarkably stable and, when dried from the frozen state and stored at low temperatures, retain their activity undiminished for many months, the dehydrogenases, on the other hand, are extremely labile and maintain their activity for relatively short periods of time. In order to rule out as far as possible the factor of enzyme lability, only those lots of freshly prepared virus which showed high infective titers were used in these studies. The methods employed for the quantitative determination of the enzymes in elementary bodies of vaccinia were tested on substrates and enzyme preparations of known purity and found to yield satisfactory results before being applied to studies on the enzyme constituents of the virus. Because of the limited quantity of virus available, certain of the methods had to be modified in order to permit quantitative determinations on small amounts of material.

Tests for Dehydrogenase Activity

Macfarlane and Salaman were unable to demonstrate dehydrogenase activity in their preparations of elementary bodies by the usual Thunberg technique at pH 7.2 and 37°C. There are certain disadvantages inherent in the use of the Thunberg technique, however, in that it is essentially a time reaction, with the end point depending on recognizable decolorization of a dye. Moreover, certain dehydrogenases may show marked inhibition by the concentration of methylene blue, or other dyes of suitable potential, which it is necessary to use in order to demonstrate reduction (7). Recently Quastel and Wheatley (8) have devised a method for the determination of dehydrogenase activity which differs materially from the Thunberg technique, and which lends itself to the use of smaller quantities of material. This method depends on the fact that electrons from hydrogen, which is taken up by coenzyme in the presence of a given dehydrogenase and its appropriate substrate, are transferred to a suitable metallic acceptor, such as ferric ion. The hydrogen ions, produced by this electron transfer, react in turn with bicarbonate buffer to yield carbon dioxide, which can be measured in the Warburg manometer. By the simple process of changing substrates, tests may be made with relative ease for a large series of dehydrogenases and the results expressed quantitatively in terms of carbon dioxide evolution. By means of this method, elementary bodies of vaccinia have been tested for malate, succinate, lactate, and pyruvate dehydrogenase activities.

Malate Dehydrogenase—10 mg. of freshly prepared virus were placed in Warburg flasks and suspended in a mixture of 0.15 cc. of 0.2 molar sodium malate and 1.35 cc. of 0.9 per cent sodium chloride containing coenzyme prepared from red blood cells. To the side arm receptacle of the flask were added 0.1 cc. of 0.03 molar sodium cyanide, 0.4 cc. of 0.16 molar sodium bicarbonate, and 0.2 cc. of 10 per cent sodium ferrocyanide. A small stick of yellow phosphorus was placed in the center inset to insure anaerobiosis, and the flasks were equilibrated with pure nitrogen gas. Appro-

priate controls of reagents with saline replacing the virus, were set up at the same time. The flasks and contents were brought to 37°C. in the water bath, and the shaker set at 100 per minute. When temperature equilibrium had been achieved the contents of the side arm were tilted into the reaction flasks and manometric readings taken at 10 minute intervals. No evolution of carbon dioxide over that in the controls without virus, was observed at the end of 90 minutes and the experiment was discontinued. Three lots of virus were tested for malic dehydrogenase with negative results in each case.

Succinate, Lactate, and Pyruvate Dehydrogenases—Tests were performed for succinate, lactate and pyruvate dehydrogenases after the manner described for malate dehydrogenase, with the substitution of 0.2 molar sodium succinate, lactate, and pyruvate, respectively, for sodium malate. Tests for each of these dehydrogenases were performed on two lots of virus with negative results in each instance. If the virus initially possessed dehydrogenase activity toward any of the four substrates tested, such activity was very likely destroyed by the washing and centrifuging processes to which the virus was subjected during purification. Tests for other dehydrogenases were not performed. Macfarlane and Dolby (9) however, report negative tests on elementary bodies for triosephosphate and hexosemonophosphate dehydrogenases. These authors likewise were unable to obtain evidence of lactate dehydrogenase.

Tests for Phosphatase Activity

Macfarlane and Salaman tested seven preparations of elementary bodies for phosphatase activity (6) with positive results in every case. Elementary bodies prepared in our laboratories were found likewise to exhibit phosphatase activity, and to an extent approximating that reported by these authors for their material. Our studies have been extended, however, and show, we believe, that the presence of phosphatase in the elementary body may well be accounted for by its having been adsorbed from tissue detritus, and that it is not, as these authors suggest, necessarily to be regarded as an integral part of the virus.

β -Glycerophosphatase Activity—To 5 mg. of freshly prepared elementary bodies were added 5 cc. of a β -glycerophosphate-buffer mixture containing a final concentration of 0.066 molar sodium barbital. The pH of the mixture was 8.9 as determined by the glass electrode. An appropriate control of pancreatic phosphatase was set up at the same time. The mixture was incubated for 1 hour at 37°C. and 0.5 cc. of 10 per cent trichloroacetic acid was added to stop enzymatic action and to precipitate the virus. The mixture was then centrifuged and inorganic phosphorus determined in the supernatant fluid by the method of Fiske and Subbarow (10). The results of the determination of phosphatase activity on four lots of elementary bodies are given in Table I. The activity is expressed in terms of milligrams of phosphorus hydrolyzed per milligram of virus per hour. The results although somewhat more constant from lot to lot, agree fairly well within a given pH range with those reported by Macfarlane and Salaman.

Adsorption of Phosphatase by Elementary Bodies of Vaccinia—The presence of phosphatase in elementary bodies of vaccinia means either that the enzyme is an integral part of the virus, or that its presence in the virus is to be accounted for on the basis of adsorption of the enzyme from the host tissues, from which the virus was separated in the process of purification. The latter hypothesis seems likely when it is considered that large quantities of ruptured white cell elements and tissue detritus, rich in phosphatase, accompany the virus in its initial stages of preparation. This set of conditions, together

TABLE I
Phosphatase Activity of Elementary Bodies of Vaccinia

Lot	Virus taken for determination	Phosphorus hydrolyzed	Phosphatase activity mg P/weight of virus
	mg	mg	
1	5	0.240	0.048
2	5	0.250	0.050
3	5	0.265	0.053
4	5	0.245	0.049

TABLE II
Phosphatase Activity of Elementary Bodies of Vaccinia Which Have Been Suspended 1 Hour in Dilute Phosphatase Solution and Washed with Buffers of Varying pH

Lot	Virus taken for determination	Phosphorus hydrolyzed	Phosphatase activity mg P/weight of virus
	mg	mg	
1	5	0.618	0.121
2	5	0.510	0.102
3	5	0.780	0.156
4	5	0.492	0.098

with the large surface area possessed by the elementary body, renders the adsorption hypothesis highly tenable. To test this possibility, elementary bodies of known phosphatase content were suspended in a dilute solution of purified pancreatic phosphatase, washed repeatedly with buffer solutions of varying pH, and retested for phosphatase activity.

Phosphatase, partially purified by fractional precipitation from a sodium chloride extract of dried pancreatin, was dried from the frozen state and the activity found to be approximately ten times that of the original pancreatin, on the basis of dry weight. 1 mg. of the crude phosphatase was dissolved in 10 cc. of 0.9 per cent sodium chloride, with the formation of a perfectly clear solution. Elementary bodies, in 5 mg. lots, were suspended in 5 cc. of this solution and allowed to remain for 1 hour at 20°C. At the end of this period the virus was recovered by centrifugation, washed alternately

by centrifugation and resuspension in successive 5 cc. portions of buffer solutions of pH 6 and 8, respectively. Four individual washings were performed, two with each buffer, and the virus resuspended in 5 cc. of the β -glycerophosphate substrate-buffer mixture previously described. At the end of a 1 hour period of incubation at 37°C. inorganic phosphorus determinations were made. The results are recorded in Table II.

The virus, in some instances, showed a threefold increase in phosphatase value after treatment with a dilute solution of crude phosphatase. That the increase in phosphatase activity was real, and not an apparent one from phosphatase which might have been carried over in solution by dilution in the washing process, was shown by the failure to obtain measurable phosphatase activity in the third and fourth buffer solutions from which the phosphatase-treated virus was recovered. This experiment indicates that the phosphatase activity shown by purified elementary bodies of vaccinia may well be due to adsorption, and that failure to remove the enzyme by repeated washing is not sufficient evidence to prove that it was an integral part of the virus. On the other hand, it does not constitute positive evidence that the phosphatase activity originally shown by the virus was due solely to adsorption of enzyme. At the moment an experiment which would offer an unequivocal solution of this problem does not suggest itself.

Catalase Activity of Elementary Bodies

In our laboratory all preparations of elementary bodies tested for catalase have thus far yielded positive results. This fact is not surprising when it is considered that the virus is in contact with tissue detritus and cell fragments, rich in catalase, in its earlier stages of preparation. That the enzyme is not carried over by dilution of tissue juices accompanying the virus has been shown by the fact that the successive buffers in which the virus is washed in the course of its purification yield negative tests for catalase. In order to show that the catalase is an integral part of the virus, however, it is first necessary to rule out the possibility of adsorption. This, as the following experiment shows, is difficult to do. The fact that elementary body preparations will adsorb and hold tenaciously large amounts of catalase from dilute solutions makes tenable the explanation that the catalase in the purified virus has been adsorbed.

Determination of Catalase.—2 cc. of a 0.1 per cent hydrogen peroxide solution in 0.15 molar potassium acid phosphate buffer, pH 7.0, were placed in a Warburg respiration flask. To the side arm were added 5 mg. of elementary bodies suspended in 0.5 cc. of dilute phosphate buffer. Appropriate controls, with buffer solution replacing the virus, were set up at the same time. The flasks were attached to the manometers and shaken in a water bath at 20°C. When temperature equilibrium had been achieved the virus was tilted from the side arm into the reaction flask and

changes in oxygen tension read at 5 minute intervals. The results for five preparations of elementary bodies are recorded in Table III. The catalase activity has been expressed as the *Kat f* value, which is the conventional term for ratio of the velocity constant, *k*, to the dry weight of the test material, or virus, on which the determination was performed.

The velocity constants, and the consequent *Kat f* values, for our preparations are significantly lower than those reported by Macfarlane and Salaman. It is possible that this discrepancy is to be explained by certain differences in the methods used for the determination of catalase activity. As may be seen from Table III, different lots of elementary bodies vary widely in their catalase content. Lot 3, for example, shows over ten times the activity of lot 2. This may be explained in part by the lability of catalase to the conditions imposed by the process of virus purification, and perhaps in part by the differences in catalase content of the cell fragments from which the virus was prepared.

TABLE III
The Catalase Activity of Purified Elementary Bodies of Vaccinia

Lot	Virus taken for determination	<i>k</i> (velocity constant)	Catalase activity <i>Kat f</i> (<i>k</i> /gm. of virus)
	mg		
1	5	0.00173	0.346
2	5	0.00087	0.174
3	5	0.00903	1.810
4	5	0.00130	0.260
5	5	0.00260	0.520

Adsorption of Catalase by Elementary Bodies of Vaccinia—Catalase was prepared in crystalline form from ox liver by the method of Sumner and Dounce (11). A solution of the crystals was diluted so that 1 cc. gave an oxygen uptake, with a hydrogen peroxide substrate in the Warburg flask, of approximately 3 to 4 cm. per minute. 5 mg. of elementary bodies, of known catalase activity, were suspended in 5 cc. of this solution for 1 hour at 20°C. At the end of this period the virus was collected by centrifugation and washed repeatedly with dilute buffer until the supernatant solution gave a negative test for catalase. The virus was reclaimed by centrifugation and dried from the frozen state. 2 mg. quantities of the catalase-treated virus were set up in the Warburg apparatus, and the oxygen tension developing from the catalase action on hydrogen peroxide determined in the manner previously described. The activity was so great as to render accurate rate determinations impossible. Within 1 minute a tension of 200 cm. and more of oxygen was developed. A determination of the velocity constant at this rate of oxygen evolution was impossible.

The experiment showed clearly that the virus possessed great surface affinity for catalase, and that relatively large amounts of the enzyme had been ad-

sorbed, and, moreover, that it had not been removed by frequent washing. In the light of these facts, the conclusion that the original catalase action of the virus may have been due to adsorbed enzyme is hard to escape.

Lipase Activity of Elementary Bodies of Vaccinia

Most tissues are rich in enzymes capable of hydrolyzing fatty acid esters of glycerol. These enzymes, which are referred to as lipases or esterases, are extremely stable, and would be expected to withstand the washing and centrifuging procedures to which elementary bodies are subjected in the course of their purification. Lipase determinations were accordingly made on several lots of the purified virus.

Determination of Lipase Activity—A number of methods for the determination of lipase activity exist. The methods most commonly employed are based on the values obtained from the titration of the fatty acid released by the hydrolysis of suitable fatty acid esters. A manometric method devised by one of us, in which the carbon dioxide released by the reaction of the fatty acid with a bicarbonate buffer is employed as a measure of lipase activity, has been used successfully in this laboratory for some time (12). The method gives accurate reaction rates and makes use of the slope of the linear portion of the curve, obtained by plotting carbon dioxide evolution against time, as a measure of the activity of the enzyme.

5 mg. of elementary bodies, suspended in 0.5 cc. of saline, were placed in the side arm of a Warburg respiration flask. To the flask were added 1 cc. of a 10 per cent emulsion of tributyrin in water, neutralized to a phenol red end point with sodium hydroxide, and 1 cc. of a bicarbonate buffer solution, containing 4.5365 gm. of sodium bicarbonate per liter. When this mixture was equilibrated with 5 per cent carbon dioxide, and diluted 1:1 with the tributyrin emulsion, a lipase substrate of pH 7.8 was obtained. The flask was equilibrated with a stream of pure carbon dioxide until the substrate became acid to phenol red, at which time a 5 per cent carbon dioxide-95 per cent nitrogen mixture was substituted for pure carbon dioxide and equilibration continued until a 5 per cent concentration of carbon dioxide in the flask was assured. Appropriate controls on the reagents, with saline replacing the virus suspension, were set up at the same time and likewise equilibrated. The flask attached to the manometer, was then shaken in a water bath at 37 C. When temperature equilibrium had been achieved, the contents of the side arm were tilted into the reaction vessel and the increase in carbon dioxide tension read at 5 minute intervals. The results of a typical reaction are given in Fig. 1.

Five lots of elementary bodies have been tested for lipase activity with positive results in each instance. From Fig. 1 it may be seen that the hydrolysis of tributyrin by elementary bodies of vaccinia is effected readily and at a constant rate over a long interval of time.

It was next decided to test the adsorptive capacity of elementary bodies for lipase in a manner analogous to that described for the adsorption of phosphatase and catalase.

Adsorption of Lipase by Elementary Bodies of Vaccinia—Lipase was prepared by ammonium sulfate precipitation of the globulin in a 10 per cent sodium chloride extract of dried pancreatin. The crude lipase globulin obtained from the sodium chloride extract was dialyzed for 24 hours against distilled water at 4°C to remove salts and finally dried from the frozen state. The lipase activity of this preparation was approximately 25 times that of the original pancreatin. 20 mg of elementary bodies were suspended in a 0.1 per cent solution of the crude lipase preparation and allowed to remain 1 hour at 20°C. The virus was reclaimed by centrifugation and

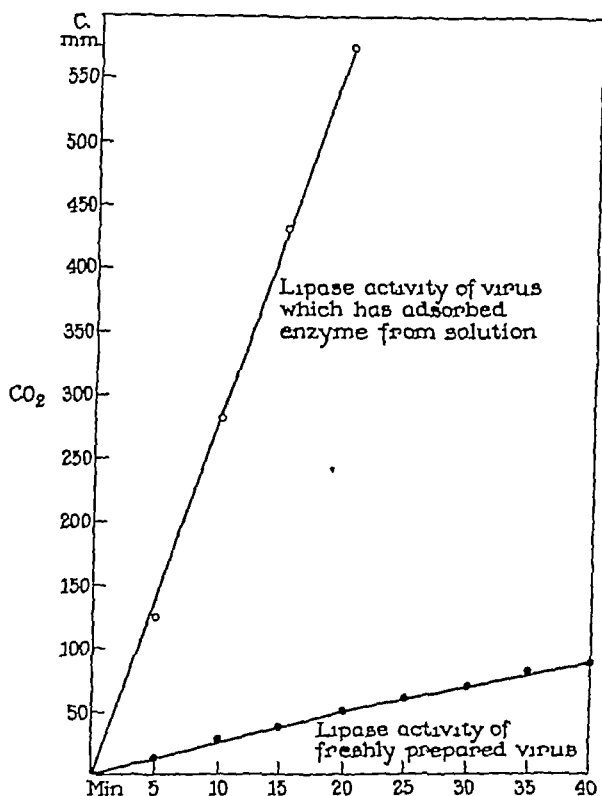


FIG 1 Hydrolysis of tributyrin by elementary bodies of vaccinia

washed by thorough resuspension and centrifugation several times in 7 to 10 cc quantities of distilled water until the supernatant waters no longer gave a test for lipase. The virus was dried from the frozen state and lipase determinations performed on 5 mg aliquots. Values for the rate of hydrolysis of tributyrin by lipase-treated virus are plotted in Fig 1.

The great capacity of purified elementary bodies to adsorb lipase is conclusively demonstrated by the foregoing experiment. From a relatively dilute solution the virus is able to effect an enormous concentration of this enzyme as shown by activity measurements before and after adsorption.

Adsorptive Specificity of Elementary Bodies

The adsorptive experiments, performed with phosphatase, catalase, and lipase show that the elementary body of vaccinia has a highly active surface capable of adsorbing certain enzymes from solution. That the adsorptive power of the virus is an exceptionally strong one is shown by the failure to remove these enzymes by frequent washing with saline and dilute buffers. It therefore became of interest to know if this property of the elementary body was exhibited indifferently to all enzymes, or if a certain degree of specificity in the adsorption could be demonstrated. Since the enzymes used in the adsorption experiments described above were of animal origin, it was of interest to know if an enzyme of vegetable origin, such as urease, might likewise be adsorbed.

Urease Adsorption Experiment with Elementary Bodies—30 mg. of elementary bodies were suspended in a 1 per cent aqueous solution of Squibb's urease for $\frac{1}{2}$ hour at 38 C. The virus was reclaimed by centrifugation and washed by resuspension and centrifugation until the supernatant wash waters no longer gave a positive test for urease. The virus was dried from the frozen state and urease determinations made on 5 mg. quantities of the dried urease-treated virus. The quantitative determination of urease activity was performed by the method of Krebs and Henseleit (13). This method was devised for the determination of urea, but can be used equally well for the determination of urease if a substrate of urea is supplied. 5 mg. of urease-treated elementary bodies were suspended in 0.5 cc. of saline and added to the side arm of a Warburg respiration flask. In the bottom of the flask were placed 1.5 cc. of a 1 per cent solution of urea and 0.5 cc. of sodium acetate-acetic acid buffer, pH 5. Enzyme controls, with 0.2 cc. of 1 per cent urease solution in the place of the virus, and reagent controls with saline replacing the virus, were set up at the same time. The flasks were attached to the manometers and shaken in a water bath at 37°C. When temperature equilibrium had been effected the virus was tilted into the reaction mixtures and changes in carbon dioxide tension read at 5 minute intervals for 1 hour. No urease activity of the treated virus could be demonstrated, although good activity of the urease preparations in the control flasks was noted consistently.

The experiments with urease show that the adsorptive capacity of elementary bodies for enzymes is to be regarded as somewhat specific. Although urease is known to have a high affinity for adsorbents, similar to that of enzymes in general, no adsorption of urease on elementary bodies could be demonstrated under the conditions of the experiment. Whether adsorption could have been demonstrated at another pH was not tested, since it was desired to learn only of the possibility of enzyme adsorption under the conditions of virus purification which is carried out at neutral pH.

DISCUSSION

The experiments described above show that elementary bodies of vaccinia, which have been prepared by washing and differential centrifugation, show

enzymatic activity toward a number of substrates. The enzymes which have been found thus far, however, are those which are known to occur in high concentration in white cells and tissue detritus. The fact that the virus is originally in intimate contact with various enzyme constituents in the dermal pulp from which the virus is prepared, makes the hypothesis that certain of these enzymes may be adsorbed on the virus, during the process of purification and concentration, a very tenable one. Dehydrogenases, and other redox enzymes which occur in cells generally and which might be expected to be adsorbed on virus particles, appear to be absent. If present initially, the lability of these enzymes might well preclude their surviving the several steps employed in the purification of the virus. Although it has not been possible to design experiments which prove that the enzymes found in the purified virus are adsorbed from tissue elements, the fact that the virus will take up large quantities of certain enzymes from a dilute solution makes a strong case for the adsorption hypothesis. Until methods can be devised which are capable of distinguishing between enzymes which represent an integral part of the virus and those which exist as contaminants, it would seem that the problem is impossible of solution. So far, no enzyme protein has been found in purified elementary bodies which is not at the same time a known constituent of normal tissue and the presence of which might not be accounted for on the basis of its adsorption from the host cells.

In the light of the above facts concerning the adsorption of enzymes by elementary bodies, what may we conclude with reference to certain other constituents which have been reported in this virus, namely, lipid, carbohydrate, nucleoprotein, copper, flavin, and biotin (1-5)? With respect to the first three substances, the fact that these occur in almost stoichiometric proportions in different preparations of virus and that they account for a major share of the mass of the virus, goes far to preclude their being accounted for on the basis of adsorption. With respect to the presence of copper, the fact that this element withstands electrodialysis, that it is released only upon hydrolysis of the virus, that it is concentrated at least 25 times in the process of virus purification, and that it occurs in constant amounts from lot to lot of elementary bodies, speaks strongly for its being an integral part of the virus. In the case of flavin adenine dinucleotide, the discovery that this substance likewise occurs in near stoichiometric relationship to other virus components, and that elementary bodies do not adsorb additional amounts of this material from a dilute solution of flavin adenine dinucleotide, speaks convincingly in favor of its close relationship to virus structure (14). As for biotin, the observation that this substance is released in relatively large amounts during the process of hydrolysis (5) of the virus likewise points to an intimate association of this material with elementary body structure. Moreover, elementary bodies show no appreciable adsorption of biotin from solution under a variety of conditions (14).

CONCLUSIONS

Purified elementary bodies of vaccinia have been tested with a variety of substrates and found to possess phosphatase, catalase, and lipase activity. Tests for malate, succinate, pyruvate, and lactate dehydrogenases were negative.

Interpretation of these results is complicated by the observation that elementary bodies of vaccinia adsorb relatively large quantities of certain enzymes from dilute solutions. These enzymes are not eluted by procedures of washing and centrifuging similar to those carried out in the preparation of the virus. For this reason, the presence of phosphatase, catalase and lipase in the purified virus may well be accounted for on the basis of adsorption from the host tissues which are known to be rich in these particular enzymes. That some degree of specificity in this adsorption is to be recognized is shown by the failure of the virus to adsorb urease, an enzyme of vegetable origin.

Until some method can be devised which will distinguish between the enzymes of the host cell and those which may be integral parts of the virus it would seem that the problem of the enzyme constituents of vaccine virus is incapable of definite solution.

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THE REACTION BETWEEN THE ENZYME TYROSINASE AND ITS SPECIFIC ANTIBODY

By MARK H. ADAMS, PH.D

(From the Hospital of The Rockefeller Institute for Medical Research)

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Tyrosinase is the enzyme which catalyses the aerobic oxidation of monohydric phenols to derivatives of catechol, and catechol derivatives to the corresponding quinones. For example, pressor substances such as tyramine and adrenalin are oxidised in the presence of tyrosinase to colored products which are physiologically inactive. Since this reaction occurs *in vitro* it was thought that tyrosinase might have a similar effect *in vivo*, and might conceivably affect experimental hypertension in animals (1) and essential hypertension in human beings (2). Because it was planned to administer tyrosinase parenterally to test this possibility two problems were presented immediately; first, is the enzyme antigenic, and second, do the antibodies if produced, affect the activity of the enzyme? This work is an attempt to answer these questions.

Previous attempts to demonstrate the production of antibodies to the enzyme tyrosinase (3-5) have failed because of the crudeness of the enzyme preparations available, and because the tests employed to indicate the production of specific antibodies were based upon the assumption that an antibody to an enzyme must neutralize its catalytic activity. Recognized immunological reactions were not used to demonstrate that antibodies had been produced. In the present work purified preparations of tyrosinase were used to immunize rabbits, and the production of specific precipitins for the enzyme tyrosinase was demonstrated.

EXPERIMENTAL

Materials and Methods—The tyrosinase used in these experiments was prepared from the domestic mushroom, *Psalliota campestris*, in the laboratory of Professor J. M. Nelson of Columbia University (6). The preparations, which closely approached the highest purity thus far obtained for this enzyme, contained about 500 catechol hydroquinone units (7) per mg. dry weight. The copper content was 0.13 per cent of the dry weight of the enzyme. Preparations available contained 3,600 catechol hydroquinone units per cc. of aqueous solution. The QO_2 value using the catechol hydroquinone substrate system was 300,000.

Immunization—Three adult male rabbits were bled from the ear, about 10 cc. of blood being taken from each for the preparation of normal control sera. These rabbits were then injected intravenously with a solution of tyrosinase in saline sterilized by passage through a Berkefeld filter. Each rabbit received 1 mg. of tyrosinase daily for 6 days, then was allowed to rest for a week. This course of treatment was

followed by two additional similar courses of injections, each rabbit thus receiving a total of 18 mg of tyrosinase over a period of 5 weeks. Ten days after the final injection of tyrosinase the rabbits were bled again and the sera tested for precipitins.

Test for Precipitation—The rabbit sera, normal and immune, were diluted with 1.5 volumes of saline. The tyrosinase at an initial concentration of 5 mg per cc or 1:200 was diluted serially with saline. Equal volumes of diluted serum and tyrosinase

TABLE I
Precipitin Titer of Normal and Immune Rabbit Sera

Rabbit serum	Concentration of tyrosinase in the mixture					
	1/400	1/1,600	1/6,400	1/25,000	1/100,000	1/400,000
Normal sera 9-93, 9-94, 9-95	—	—	—	—	—	—
Immune serum 9-93	+++	+++	++++	++++	++	±
Immune serum 9-94	+++	+++±	+++	+++	±	trace
Immune serum 9-95	+++	+++±	++++	+++	++	±

+++ = pronounced precipitation with clear supernatant.

± = faint precipitation

— = no precipitation

TABLE II
*Precipitation of Anti-*Psalliota* Tyrosinase Rabbit Serum by *Psalliota* and *Lactarius* Tyrosinases As Antigens*

Antigen used	Concentration of antigen in the mixture					
	1/1,000	1/2,000	1/4,000	1/8,000	1/16,000	1/32,000
<i>Psalliota</i> tyrosinase	+++	+++±	++++	++++	++++	++++
<i>Lactarius</i> tyrosinase	—	—	—	—	—	—

+++ = pronounced precipitation with clear supernatant

— = no precipitation

The serum used was immune serum 9-93. Experimental conditions as in Table I.

solution were mixed, incubated at 37° for 2 hours, and placed in the refrigerator overnight before reading. The results are given in Table I.

It is evident that the sera of rabbits injected with a preparation of the enzyme tyrosinase contained precipitins for the antigen used, and that such precipitins were absent from the sera of these same rabbits before immunization.

A preparation of tyrosinase from the mushroom *Lactarius piperatus* of equivalent strength and purity (8) was tested against the antiserum to the *Psalliota campestris* tyrosinase under the conditions specified above. The results of this experiment are included in Table II.

The tyrosinase from the mushroom *Lactarius piperatus* failed to show any

cross precipitin reaction with the antiserum to the *Psalliota campestris* tyrosinase.

A preparation of *Psalliota* tyrosinase which had been inactivated with regard to catalytic activity by heating at 80° for 10 minutes was also tested in the same way for precipitation with antiserum to the *Psalliota* tyrosinase. The results (Table III) indicate that a temperature sufficient to destroy the catalytic activity of the enzyme affected but little its ability to function as a specific antigen in the precipitin reaction.

Human Sera Reacting with Tyrosinase.—In connection with a study on hypertension undertaken in the Hospital of The Rockefeller Institute by Dr. Schroeder (2) patients were treated by injections of tyrosinase. The tyrosinase, dissolved in physiological saline, was passed through a Berkefeld filter and proven bacteriologically sterile before injection. Blood specimens were ob-

TABLE III
Precipitation of Heat Inactivated Tyrosinase in Anti Tyrosinase Rabbit Serum

Antigen used	Concentration of antigen in the mixture			
	1/5,000	1/25,000	1/125,000	1/625,000
Active <i>Psalliota</i> tyrosinase	++++	++++	++±	±
Heat inactivated <i>Psalliota</i> tyrosinase	++++	++++±	++	±

++++ = pronounced precipitation with clear supernatant.

± = faint precipitation.

The serum used was immune serum 9-93. Experimental conditions as in Table I

tained from some of these treated patients, as well as from normal untreated human beings, and sera were prepared. These sera were tested for tyrosinase precipitins in the manner described above for rabbit sera (Table IV).

The treatment with tyrosinase including total dosage, period of treatment, and interval between final injection and removal of blood samples, is summarized in Table V. Included in this table are observations by Dr. Schroeder regarding the severity of local or systemic reactions. It is evident from Table V that there is no correlation between the quantity of antigen administered and the serological antibody response. There is also a lack of correlation between the antibody response and the sensitivity of the patient to injections of the enzyme. The patient sometimes experienced a maximum reaction at the first injection obviously before any sensitization could take place. The reaction of the patients to injections of the enzyme will be discussed in greater detail in a paper to be published by Dr. Schroeder.

*Effect of Immune Rabbit Serum on the Catalytic Activity of *Psalliota campestris* Tyrosinase.*—Since the immune rabbit sera gave precipitin reactions with highly purified preparations of the enzyme tyrosinase at an antigen concentration of

TABLE IV

Precipitin Titers of Sera from Patients Treated with Tyrosinase and from Untreated Human Beings

Serum	Concentration of tyrosinase in the mixture			
	1/5,000	1/15 000	1/50,000	1/500,000
G.P	+	++++	+++	±
J.P	+	+	++	—
L.G	++	+	±	—
J.R.	++	+	±	—
H.H	+	±	trace	—
J.F	±	±	—	—
A.Z	±	±	—	—
J.S	—	—	—	—
R.L	—	—	—	—
A.L normal	—	—	—	—
C.M normal	—	—	—	—
Rabbit 9-93 (immunized)	+++±	++++	++++	±

++++ = pronounced precipitation with clear supernatant

± = faint precipitation

— = no precipitation

TABLE V

Administration of Tyrosinase to Patients

Patient	Total tyrosinase administered	Route of administration	Period of treatment	Interval between injection and blood sampling	Anti bodies in blood sample	Local or systemic reaction
	mg		days	days		
G.P	75	Subcutaneous	23	47	+++	None
J.P	40	"	7	11 28	++	Swelling and tenderness, slight to moderate at site of injection
L.G	5	Intramuscular	5	30	+	Shock reaction, not anaphylactic in origin
J.R	250 25	Subcutaneous "	100 9	6	+	None
H.H	18	"	4	8	±	No local reaction but high fever
J.F	55	"	20	45	±	Slight local reactions
A.Z	44	"	30	None	±	Slight local reaction
J.S	56 58	Intravenous Subcutaneous	22 15	46	—	Moderate local reaction
R.L	83 24	Intravenous Subcutaneous	14 5	15	—	None

1/500,000 it was thought that the antisera might neutralize or inhibit the catalytic activity of the enzyme. To test this possibility the mixture of tyrosinase and rabbit antiserum after the precipitin reaction had taken place was titrated for enzyme activity.

The immune serum from rabbit 9-93 was utilized since it had the highest precipitin titer. As a matter of convenience the normal serum of rabbit 9-94 was utilized as the control. The tyrosinase was so diluted with saline that the final concentration of tyrosinase in the mixture with serum was 1/20,000 the concentration at which maximum precipitation occurred as indicated in Table I. The sera employed were diluted with 1.5 volumes of saline. To 1 cc. of diluted serum was added 1 cc. of tyrosinase in saline at a concentration of 1/10,000, an amount corresponding to 45 units of catecholase activity. The serum-antigen mixtures were incubated 2 hours at 37°C. and kept overnight in the refrigerator. Three centrifuge tubes were set up as follows —

Tube 1 1 cc. normal serum 9-94 + 1 cc. of tyrosinase 1/10,000

Tubes 2 and 3 1 cc. immune serum 9-93 + 1 cc. of tyrosinase 1/10,000

TABLE VI

Effect of Homologous Antibody on the Catalytic Activity of Tyrosinase

Rabbit serum plus tyrosinase	Tyrosinase activity in catecholase units per cc. of final dilution	
Normal serum 9-94 tube 1	0.45	0.45
Immune serum 9-93 tube 2	0.05	0.05
Immune serum 9-93 tube 3	0.46	0.45

No precipitate developed in tube 1 containing the normal serum, while tubes 2 and 3 contained a flocculent precipitate. An aliquot of tube 1 was diluted 1:50 with saline. Tube 2 was centrifuged to bring down the precipitate and an aliquot of the clear supernatant was diluted 1:50 with saline. Tube 3 was well stirred to distribute the precipitate uniformly and an aliquot was diluted 1:50. No precipitate was noted in this case after dilution. The enzyme in each case had been diluted to one one hundredth of its initial concentration and hence should have had an activity of 0.45 units per cc. The catecholase activities were determined in the Warburg apparatus in the usual manner (7). The results of duplicate determinations are given in Table VI.

It is evident from this experiment that near the optimum concentration of enzyme and antibody for the precipitin reaction, as previously determined by the experiment of Table I, neither normal nor immune rabbit serum had any effect on the catalytic action of tyrosinase. If, however, the immune precipitate was removed by centrifugation, nine-tenths of the enzymatic activity was likewise removed.

In another experiment the ratio of antibody to antigen was increased 100-fold by mixing undiluted immune serum with 0.9 unit of enzyme and incubating samples of the mixture at 37° for various intervals from 15 minutes to 2

hours, after which the enzyme activities were determined. In no case did this large excess of antibody have any effect on the catalytic activity of the enzyme in the mixture.

We can conclude then, that it is valid to determine the activity of tyrosinase in the presence of antibody, and, furthermore, that the activity so determined is a correct indication of the amount of tyrosinase present in the sample under investigation.

TABLE VII

Quantitative Titration of the Precipitin Reaction between Tyrosinase and the Homologous Immune Rabbit and Human Sera

Experiment No	Enzyme added		Enzyme in supernatant	Enzyme in precipitate	Percent age of added enzyme in precipitate	Enzyme in precipitate	Total precipitate	Antibody N in precipitate	Ratio of Antibody N to Antigen N in precipitate
	mg of N	units	units	units		mg of N	mg of N	mg	
Rabbit serum									
1	0.0125	30	0.4	30	100	0.0125	0.094	0.080	6.4
2	0.0125	30	0.4	30			0.092		
3	0.025	59	0.5	59	100	0.025	0.158	0.132	5.28
4	0.025	59	—	59			0.155		
5	0.050	118	0.5	118	100	0.050	0.258	0.206	4.12
6	0.050	118	—	118			0.253		
7	0.10	235	34	201	82	0.082	0.276	0.190	2.32
8	0.10	235	51	184			0.268		
Human serum									
9	0.01	28	0.4	28	100	0.010	0.079	0.069	6.9
10	0.02	57	2.0	55	98	0.020	0.130	0.110	5.5
11	0.03	85	6.0	79	93	0.028	0.151	0.123	4.4
12	0.04	113	14.0	99	88	0.035	0.157	0.122	3.5
13	0.05	142	36.0	106	75	0.037	0.159	0.122	3.3
14	0.10	284	150.0	134	47	0.047	0.126	0.079	1.7

Quantitative Determination of Precipitins—Since evidence for the absolute purity of the tyrosinase preparation is lacking it was necessary to learn if the antibody of the rabbit serum was precipitating the tyrosinase itself and not merely reacting with an accompanying contaminant. The test involved a quantitative precipitin titration by the method of Heidelberger and Kendall (9) together with a simultaneous assay for enzymatic activity of the supernatant liquid remaining after removal of the specific precipitate. Since it has been shown above that the presence of immune serum has no effect on the determination of the catalytic activity of tyrosinase, it is legitimate to conclude that any tyrosinase not found in the supernatants must be part of the immune precipitate.

Immune rabbit sera 9-93 and 9-95 which were approximately equal in precipitin titer were pooled, and centrifuged under the conditions of the experiment to remove any sedimentable material. The human serum of highest precipitin titer for tyrosinase available (G.P. in Table IV) was treated in the same manner. The tyrosinase preparation to be used as antigen in the precipitin reactions was centrifuged also to make certain that no sedimentable material was present in the antigen solution. The Kjeldahl nitrogen content of this enzyme solution was determined, and enzyme dilutions in saline were

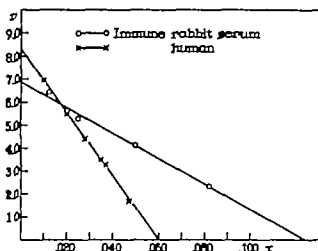


CHART 1 In this chart the variables r and x of the equation of Heidelberger and Kendall are plotted

r = ratio of $\frac{\text{Antibody N}}{\text{Antigen N}}$ in the precipitate

and x = milligrams of antigen N in the precipitate.

In the equation of Heidelberger and Kendall the constants as calculated from these curves are for the immune rabbit serum

$R = 3.4$ and $A = 0.212$ mg of N,

and for the immune human serum

$R = 4.2$ and $A = 0.126$ mg of N

made on the basis of nitrogen content. In each experiment 2 cc. of serum were diluted with 3 cc. of saline, and 1 cc. of tyrosinase appropriately diluted with saline was added. After mixing the tubes were incubated 2 hours at 37°C and overnight in the refrigerator. The precipitates were centrifuged down at 0°C, washed twice with saline at 0°C, dissolved in dilute NaOH, and transferred to Kjeldahl flasks for nitrogen analysis. The clear supernatant fluid, decanted from the precipitate after centrifugation, was titrated for tyrosinase activity in the Warburg apparatus. The second saline washings of the precipitates contained no measurable enzyme activity indicating that the immune precipitates did not dissociate appreciably in physiological saline.

Since the nitrogen content of the added tyrosinase is known, and since the

tyrosinase remaining in the supernatants can be determined, the amount of tyrosinase nitrogen present in the specific precipitates can be calculated by difference. The total nitrogen content of the specific precipitate is known from the Kjeldahl determinations. Consequently the amount of antibody nitrogen present in the immune precipitates can be calculated by difference. Then knowing the amounts of both antigen and antibody nitrogen in the immune precipitates it becomes possible to apply the empirical relationship of Heidelberger and Kendall (9) to our results.

The equation of Heidelberger and Kendall is

$$r = 2R - \frac{R^2 X}{A}$$

in which

$$r = \text{ratio of } \frac{\text{Antibody N}}{\text{Antigen N}} \text{ in precipitate}$$

$$X = \text{milligrams of antigen N in precipitate}$$

$$R = \text{ratio of } \frac{\text{Antibody N}}{\text{Antigen N}} \text{ at equivalence point}$$

$$A = \text{total antibody present in milligrams of N}$$

As can be seen from Chart 1 both human and rabbit immune sera satisfy the empirical equation of Heidelberger and Kendall even in the region of antigen excess where the reaction is in the post zone. As can be seen from the constant A of the equation the rabbit serum contained a much greater quantity of antibody than did the human serum.

DISCUSSION

The sera obtained from rabbits injected with tyrosinase precipitate tyrosinase activity from solution quantitatively. Similarly the serum obtained from a human being injected with tyrosinase precipitated this enzyme completely from solution. Since the reaction between these sera and the antigen, tyrosinase, follows the same quantitative equation which has been applied to many other precipitin reactions, it is felt that the production of precipitating antibodies to the enzyme tyrosinase in rabbits and in human beings has been demonstrated. The enzyme tyrosinase is antigenic in rabbits and human beings, but the antibodies produced, though precipitating the enzyme do not neutralize its catalytic activity.

The results of immunization of the three rabbits were very uniform. However, in the case of human beings injected with tyrosinase there was wide variation in antigenic response (Table IV) and no correlation was evident between antibody production and dosage or extent of treatment. There was, moreover,

no correlation between the amount of circulating antibody at the time of blood sampling and the severity of local reaction at the site of injection or of systemic reaction to the injection of tyrosinase

There is in the literature adequate evidence for the production of antibodies against only a few enzymes. Sumner and Kirk (10) prepared an immune rabbit serum which gave a precipitin reaction with jack bean urease at an antigen concentration of one part in 600,000. Campbell and Fourt (11) prepared an immune rabbit serum which gave a positive ring test at a catalase concentration of one part in 300,000. Seastone and Herriott (13) prepared an immune rabbit serum which gave a positive ring test at a pepsin concentration of one part in 100,000. They also prepared an immune serum which reacted with pepsinogen diluted one part to 1,000,000. During the present work immune rabbit serum has been prepared which gives a precipitin reaction at a tyrosinase concentration of one part in 500,000.

Sumner found that the specific precipitate exhibited 80 per cent of the catalytic activity of the urease present. The 20 per cent loss of activity was ascribed to the lowered degree of dispersion of the enzyme in the immune precipitate. Campbell and Fourt found that the immune precipitate between antibody and catalase was just as active as the enzyme alone would have been. Similar observations cannot be made in the case of pepsin since this enzyme is inactivated at the pH of serum, or in the case of pepsinogen which is an enzyme precursor. In the case of tyrosinase the immune precipitate exhibits all the catalytic activity of the precipitated enzyme.

Kirk found that the rabbit antibody to jack bean urease also was able to precipitate soy bean urease (14). Campbell and Fourt found that serum prepared from rabbits immunized to crystalline beef liver catalase also precipitated horse and dog liver catalase (11). Tria found that a similar rabbit antiserum to beef liver catalase likewise precipitated lamb liver catalase (12). Seastone and Herriott immunized rabbits to swine pepsin and found that the resultant antiserum precipitated beef and guinea pig pepsins as well as swine pepsin, but did not precipitate rabbit, shark, or chicken pepsins. In the present work it was shown that rabbit antiserum against *campestris* tyrosinase failed to precipitate the tyrosinase prepared from the *Lactarius piperatus* mushroom.

It is evident in the cases of urease, catalase, and tyrosinase that the portion of the molecule involved in catalytic reaction with the substrate is not identical with the portion of the molecule involved in the reaction with the serum antibody. The enzyme molecule is capable of reacting simultaneously with two different substances. Similar observations have been reported in the case of antipneumococcus horse serum proteins which can react simultaneously with pneumococcal capsular polysaccharide and with anti-horse chicken serum (15) and also in the case of antigenic antitoxins. We must conclude that when a

physiologically active substance is used as an antigen the production of antibodies must be tested by recognized immunological reactions such as the precipitin reaction, as well as by possible neutralization of the physiological activity of the antigen

SUMMARY

1 Antibodies to the enzyme tyrosinase, obtained from the mushroom *Psalliota campestris*, have been produced in rabbits and human beings

2 These antibody preparations, though precipitating the enzyme from solution, do not affect its catalytic activity

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A NON VIRULENT, SINGLE-DOSE RABIES VACCINE FOR PROPHYLACTIC IMMUNIZATION OF DOGS

By L. T. WEBSTER, M.D., AND J. CASALS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Experiments have been described indicating that a vaccine containing approximately 50,000 mouse intracerebral lethal doses of rabies virus properly irradiated will immunize mice, and that 25,000,000 doses will immunize dogs against a subsequent test inoculation of virulent virus. Additional experiments showed that a volume of 0.1 cc. of an irradiated vaccine prepared from the supernatant of centrifuged virus-containing dog brain contained the required 50,000 doses and immunized mice adequately (1). It remained to determine whether the required number of mouse doses to immunize dogs could be concentrated into a small volume and whether a single injection of such a vaccine would in fact protect them adequately.

Technique

To prepare vaccine, 2 months-old beagle dogs are injected intracerebrally with 0.5 cc. of a 1:10 dilution of dog brain virus, Pasteur strain. When the animals become prostrate, they are sacrificed, their brains are removed, triturated in an electric mixer and diluted in distilled water containing 2 per cent citric acid-sodium diphosphate buffer (McIlvaine), pH 7.2—to make a 1 or 5 per cent emulsion. This emulsion is then centrifuged at 500 R.P.M. for 5 minutes and the supernatant removed. The supernatant must be relatively free from large particles to insure that ultraviolet light will render it non virulent and yet not destroy its immunizing potency. It must also titre at least 330,000 mouse doses per mL., that is, prove fatal to W Swiss mice when injected intracerebrally in 0.03 cc. volumes and in a dilution of 10^{-4} to insure that small volumes of the final vaccine will immunize animals effectively. The material is rendered non virulent by exposure to ultraviolet light for 35 minutes (2). Tests for sterility are made, merthiolate in a dilution of 1:10,000 is added as preservative, and the material ready for use, is stored in the ice box at 40°F.

The technique which we have employed for testing rabies vaccines in dogs has been described in a previous paper (3). Beagle dogs aged 4 to 6 months are used for the most part in all tests. In some, however, it has been necessary to include animals of mixed small breeds of the same age. They are wormed as soon as received on the premises and then given 10 to 15 cc. of distemper immune serum followed by one or two injections of non virulent distemper vaccine.

About 7 days after arrival, groups of six to twelve animals receive rabies vaccine and a remaining group is set aside as controls.

Three weeks later all are given a test injection of rabies virus 0.25 cc. properly diluted deep into the neck muscles of each side. They are then watched for signs of

rabies for 2 to 5 months. The animals which become prostrate are sacrificed, their brains removed, tested for the presence of Negri bodies in stained impressions, and for the presence of rabies virus by inoculation of diluted brain suspension intracerebrally into mice. The survivors are also sacrificed when the experiment is terminated and their brains tested for the presence of rabies virus.

Results of Tests on Unvaccinated Dogs

In attempting to evaluate experiments on the immunizing effects of vaccines on experimental animals, a factor of prime importance is the regularity of response of unvaccinated controls to the test virus. When small animals, such as mice, are employed, it is possible to exert such a degree of control that not only unvaccinated animals respond in a uniform and predictable manner within narrow limits, but the actual amount of virus administered can be determined in terms of minimum lethal doses. When large and expensive animals are employed, such as the monkey or dog, the problem becomes more difficult, although the aims remain the same, namely, to set up conditions which will insure uniform and predictable mortalities among unvaccinated animals.

These aims have been realized in part only. English investigators in India (4, 5), studying the potency of rabies vaccines in monkeys, found that mortalities in unvaccinated groups following the test injection of virus varied from 20 to 100 per cent in different experiments. More recently, Johnson and Leach (6) carried out repeated tests of vaccines in thirteen groups of seven to ten dogs and found that the mortalities among unvaccinated dogs varied unpredictably from 20 to 91 per cent. They attribute such "fallacious results" to chance and carried out repeated tests until the total number of animals "satisfied statistical requirements" (6). They also performed a single experiment with fifty-five control dogs which they regarded as sufficient to eliminate the effects of chance variables encountered in their type of test. We have been more fortunate in controlling chance variables in our experiments with smaller groups of animals and have obtained results in unvaccinated dogs which show little variation from experiment to experiment. In six tests shown in Table I, the mortalities among the unvaccinated dogs averaged 83 per cent and were never less than 71 nor more than 88 per cent except in one instance in which the mortality was 100 per cent. These results have been similar in all eighteen tests in that the controls showed mortalities as great as 100 per cent but never less than 71 per cent except on two occasions when mortalities were 60 and 50 per cent respectively. The duration of life of these animals varied widely: 39.6 per cent in 12 to 19 days, 27 per cent in 20 to 29 days, 16.6 per cent in 30 to 60 days, and 16.6 per cent remained well more than 90 days. This means that 66.6 per cent of the animals died within the first month. Under natural conditions of street infection, probably not more than 20 per cent die during a similar period.

Although we have been successful in achieving relatively uniform and pre-

dictable results in mortalities following injection of groups of five to eleven control dogs with rabies virus, we have not been successful in eliminating completely complicating factors of intercurrent infection—in our case, distemper. The British workers referred to above lost as many as 20 per cent of their animals from intercurrent infections—chiefly among the vaccinated groups—and hence compared vaccinated and unvaccinated groups at 2 rather than 3 months following infection. Leach and Johnson, in their thirteen tests with small batches of dogs, lost not only 49 per cent of the original 149 controls from the experimental inoculation with rabies virus, but also 19 per cent from other

TABLE I

Mortality of Unvaccinated Control Dogs, Tests 10-18 Following Intramuscular Inoculation of Virus

Test	Virus	Dilution	No. of dogs dying from rabies on given days following inoculation																Dead/ Total	Dead per cent			
			12	13	15	16	17	18	19	20	21	23	24	25	27	28	30	34			37	41	Survivors
10	Fixed No. 15811	1 4 000			1	1		1									1				1	4/5	80 0
12	Fixed No. 15811	1 400				2	1		1	1				1			1		1		2	9/11	81 8
13	Fixed No. 15811	1 400			1		1	2		1						1		1			2	7/9	77 7
15	Fixed No. 15811	1 200										3	1			1					2	5/7	71 4
16	Street (1 passage)	1 20					1			1				1	1		2		1	1	0	8/8	100 0
18	Street (1 passage)	1 20	1	4					2												1	7/8	87.5
			19/48—39 6 per cent 12 to 19 days				13/48—27 per cent 20 to 29 days				8/48—16 6 per cent 30 to 60 days				8/48—16 6 per cent Survived				40/48		83 3		

causes, they also lost 18.6 per cent of the original 140 vaccinated dogs from the experimental inoculation and an additional 25 per cent from other causes. The effect of uncontrolled variables was therefore equal to 40 per cent of, or even exceeded, the total effect of the test inoculation and was, therefore, highly selective. From the biological viewpoint, it is questionable whether results with such selected animals are completely reliable. Bearing this in mind, we have chosen to present for main consideration only those tests on vaccines in which mortalities from other causes have been not greater than 5 per cent of the original population.

Results of Tests on Vaccinated Dogs

The first experiment was planned to determine whether a calculated amount of irradiated vaccine given in a single dose intraperitoneally would immunize

dogs and, at the same time, whether the immunity obtained would compare favorably with that achieved by commercial canine vaccines

Experiment 1, Test 10, October 16, 1939—A 1 per cent mouse brain irradiated vaccine was prepared as described above. Prior to irradiation the 1 per cent vaccine titred 330,000 mouse doses per cc. 8 days later, ten dogs, groups B and D, were each given 40 cc. of this vaccine in a single dose intraperitoneally and 5 dogs, group E, were each given 10 cc. An additional five dogs, group F, were each given a single 5 cc. dose of a commercial 33 per cent chloroformized canine vaccine subcutaneously, and still

TABLE II
Immunizing Effects of Antirabies Vaccines on Beagle Dogs
Experiment 1, Test 10

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus No 15811		
		Day of death following inoculation	No dead/No injected	Dead
A No vaccine	1 400	13,20,S	2/3	66
B Vaccine irradiated, 40 cc, intraperitoneally	"	S,S,S,S	0/4	0
C No vaccine	1 4,000	15,16,18,30,S	4/5	80
D Vaccine irradiated, 40 cc, intraperitoneally	"	53,S,S,S,S	1/5	20
E Vaccine irradiated, 10 cc, intraperitoneally	"	S,S,S,S,S	0/5	0
F Vaccine chloroform, 5 cc, subcutaneously	"	15,19,S,S,S	2/5	40
G Vaccine phenol, 5 cc, subcutaneously	"	14,16,17,43,S	4/5	80
H No vaccine	1 40,000	12,39,S	2/4	50

S = animal remained well following injection. Survivors discarded after 70 days

another five dogs, group G, a single 5 cc. dose of a commercial phenolized canine vaccine subcutaneously. Twelve remaining dogs were set aside as controls. 3 weeks later, all save one dog in group B, which died of distemper, were ready for the test inoculation of virulent virus. A strain of dog passage virus, No 15811, which had been employed in previous tests (2), was prepared as described above and given into the neck muscles of the dogs in the following manner. Group B, plus a group of three controls (group A), received an injection of 0.25 cc. of a 1 400 dilution into the neck muscles of each side, groups D, E, F, and G, plus five controls (group C), the same inoculation of a 1 4,000 dilution, and four controls (group H), a 1 40,000 dilution. Animals were observed 70 days.

The results of this test are shown in Table II. Of greatest interest are the animals receiving the 1 4,000 dilution of virus which, in previous tests, has

been shown to produce rabies in 50 per cent and less than 100 per cent of controls and to contain roughly about ten lethal doses for dogs (3). In the present test, this amount of virus was fatal to four of five unvaccinated dogs (80 per cent), (group C), and one tenth of this amount was fatal to two of four unvaccinated dogs (group H). The 1:4,000 dilution of virus was likewise fatal to four of five dogs receiving phenolized vaccine (80 per cent), (group G), to two of five receiving chloroformized vaccine (40 per cent), (group F), but to only one of five receiving 40 cc. of irradiated vaccine (20 per cent), (group D), and to none of five receiving 10 cc. of the irradiated vaccine (group E). Finally ten times this dose of test virus was fatal to two of three controls (66 per cent), (group A), but to none of four dogs receiving 40 cc. of irradiated vaccine (group B). Clearly the irradiated vaccine in 40 or 10 cc. amounts conferred a strong

TABLE III
Immunizing Effects of an Irradiated Antirabies Vaccine on Beagle Dogs
Experiment 2 Test 13

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of dog passage virus No. 15811		
		Day of death following inoculation	No. dead/ No. injected	Dead per cent
A. No vaccine	1:400	15, 17, 18, 18, 20, 28, 34, S, S	7/9	77.7
B. Vaccine irradiated 10 cc. intraperitoneally	"	17, 19, 24, 29, 31, 38, S, S, S, S	6/10	60.0
C. Vaccine irradiated, 2 doses 10 cc. each, intraperitoneally	"	17, 17, S, S, S, S, S, S	2/8	25.0

immunity to thirteen of fourteen dogs, whereas phenolized vaccine failed and chloroformized vaccine succeeded only partially in immunizing the animals.

Inasmuch as 10 cc. or more of irradiated vaccine immunized dogs successfully in Experiment 1, the test was repeated using 10 cc. or 20 cc. of vaccine per dog.

Experiment 2 Test 13, April 8, 1940—A 1 per cent dog brain irradiated vaccine was prepared in the manner described above. Prior to irradiation 1 per cent vaccine titred 3,300,000 mouse doses per cc. 15 days later ten dogs, group B, were given a single dose of 10 cc. of the vaccine intraperitoneally, and eight dogs, group C, two doses of 10 cc. 1 week apart. Ten dogs group A were set aside as controls. 3 weeks later all dogs were given the stated inoculation of virulent dog passage virus strain 15811 diluted 1:400 and observed for 70 days.

The results of this test are shown in Table III. One of the control dogs (group A) died of distemper on the 22nd day. Of the remaining nine seven died of rabies (77.7 per cent). Six of ten dogs receiving 10 cc. of vaccine (group B) also died of rabies (60 per cent), whereas only two of eight receiving

two 10 cc doses of vaccine (group C) succumbed (25 per cent) Evidently in this test 10 cc of vaccine produced little immunity and 20 cc. a good immunity

In the following experiment a larger amount of irradiated vaccine was employed—30 cc in a single dose and in three doses of 10 cc each

Experiment 3, Test 16, October 10, 1940—A 1 per cent dog brain virus was prepared and tested as in the previous experiment. Its titre prior to irradiation was 3,300,000 mouse doses per cc 9 days later eight dogs were given a single injection of 30 cc of vaccine intrapentoneally (group B), and eight dogs three injections of 10 cc each (group C) Eight dogs (group A) were reserved as controls 3 weeks later, all but one dog in group B and one in C, which died of distemper, were inoculated into the

TABLE IV
Immunizing Effects of Irradiated Anthrabies Vaccine on Beagle Dogs
Experiment 3, Test 16

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of one-passage virus No 17456		
		Day of death following inoculation	No dead/ No injected	Dead
A No vaccine	1 20	17,20,25,27,30,30,37,41	8/8	per cent 100
B Vaccine irradiated, 30 cc, intrapentoneally	"	S,S,S,S,S,S,S	0/7	0
C Vaccine irradiated, 3 doses, 10 cc each, intrapentoneally	"	S,S,S,S,S,S,S	0/7	0

neck muscles with one-passage street virus, No 17456, diluted 1 20 The animals were observed for rabies for 70 days

The results of this test, shown in Table IV, were clear-cut All of the controls died of rabies but none of the fourteen vaccinated dogs A total of 30 cc of irradiated vaccine either in single or multiple doses conferred a strong immunity, as already indicated in Experiment 1

It was apparent from these tests that 25 to 30 cc of 1 per cent irradiated vaccine would immunize young beagle dogs against an inoculation of fixed or street virus fatal to nearly all unvaccinated dogs It remained to discover whether the same amount of vaccine concentrated to a smaller volume would be equally effective as an immunizing agent

Experiment 4, Test 18, May 27, 1941—A 5 per cent rather than a 1 per cent supernatant dog brain virus was prepared as a vaccine, thus effecting at once a 5 times concentration of virus per cubic centimeter This material titred 33,000,000 mouse doses per cc, as did preparations in other tests prior to irradiation Following irradiation,

the 5 per cent vaccine was concentrated 6 times at low temperature *in vacuo* 27 days later eight dogs (group B) were given a single dose of 5 cc. intraperitoneally, eight dogs (group C) 5 cc. subcutaneously, eight dogs (group D) two injections of 5 cc. each intraperitoneally, and eight dogs (group E) 5 cc. of a commercial chloroformized vaccine subcutaneously. Eight dogs were reserved as controls. 3 weeks later, all received the standard inoculation into the neck muscles of one-passage street virus No. 17825, diluted 1:20. The animals were observed 70 days for signs of rabies.

The results of this test were again striking (Table V). Seven of the eight control dogs (group A) died of rabies in 12 to 19 days (87.5 per cent), indicating an unusually virulent street virus. Three of the eight dogs given chloro-

TABLE V
Immunizing Effects of Irradiated Antirabies Vaccine on Beagle Dogs
Experiment 4 Test 18

Treatment of dogs	Dilution of test virus	Fate of dogs inoculated into the neck muscles (right and left) with 0.25 cc. of one-passage street virus		
		Day of death following inoculation	No. dead/ No. injected	Dead per cent
A No vaccine	1:20	12, 13, 13, 13, 19, 19, 19, 19 S	7/8	87.5
B Vaccine irradiated, concentrated 5 cc., intraperitoneally	"	S, S, S, S, S, S, S, S	0/8	0.0
C Vaccine irradiated concentrated, 5 cc., subcutaneously	"	S, S, S, S, S, S, S, S	0/8	0.0
D Vaccine irradiated concentrated 2 doses, 5 cc. each intraperitoneally	2	S, S, S, S, S, S, S, S	0/8	0.0
E Vaccine chloroform, 5 cc. subcutaneously	"	12, 13, 19, S, S, S, S, S	3/8	37.5

formized vaccine (37.5 per cent) likewise died of rabies (group E), whereas all of the twenty-four dogs given irradiated vaccine remained well. It appears, therefore, that 5 cc. or more of this concentrated vaccine protects highly susceptible dogs against rabies.

Having demonstrated that a single 5 cc. dose of our non virulent rabies vaccine immunized dogs effectively, we sought information from all of our tests as to (1) the comparative effectiveness in dogs of irradiated, phenolized, and chloroformized vaccines, and (2) the comparative effectiveness of the intraperitoneal as opposed to the subcutaneous route of injecting the vaccine.

With respect to the relative immunizing potencies of irradiated as compared to phenolized or chloroformized vaccines, the following information is at hand. Summarizing all data in nineteen dog tests (Table VI), we noted that the mortality of 141 controls given test virus in dilutions corresponding to those given

to vaccinated animals was 92.2 per cent. Similarly, the mortality of fifty-three dogs given commercial phenolized vaccine according to directions was 77.3 per cent and that of fifty dogs given commercial chloroformized vaccine, 46.0 per cent. In contrast, of thirty-eight dogs given 20 to 40 cc of irradiated vaccine intraperitoneally, 13.1 per cent succumbed, and of thirty-one given 5 to 10 cc of irradiated vaccine, concentrated, only one succumbed to the test inoculation of virulent virus. Clearly the course of vaccination with irradiated vaccine appears far superior to that of vaccination with commercial vaccines.

From the figures of Table VI alone, however, one would rightly question whether the superior results with irradiated vaccine were due only to the quality of the material, or to the greater amounts employed and to the routes of inoculation. It is our belief that all three factors contribute to the potency of the

TABLE VI

Comparison of Commercial (Phenolized, Chloroformized) and Irradiated Canine Antirabies Vaccines

Groups		Total dead/ Total tested	Dead <i>per cent</i>
A	Controls Fixed virus, dilution 1:50 to 1:10,000, street virus, dilution 1:20	130/141	92.2
B	Vaccinated Phenolized vaccine, 5 cc, subcutaneously	41/53	77.3
C	Vaccinated Chloroformized vaccine, 5 cc, subcutaneously	23/50	46.0
D	Vaccinated Irradiated brain tissue supernatant		
	1 20-40 cc, intraperitoneally	5/38	13.1
	2 5-10 cc concentrated, intraperitoneally	1/31	3.2

irradiated vaccine and the following reasons are emphasized. That irradiated vaccine given intraperitoneally is superior to equal quantities of chloroformized vaccine given by the same route is indicated by the quantitative results of three experiments (1) in which one part of a single pool of virus material was inactivated by chloroform and another part by irradiation. Each inactivated preparation was then given to mice as vaccine and in each instance the irradiated sample proved equal in immunizing potency or superior to the chloroform-treated sample. In further tests, the irradiated vaccine was compared with commercial canine vaccines for ability to immunize dogs. The results shown in Table VII, though not critical, confirm the findings in mice. The vaccines contained widely different amounts of brain-virus—33 per cent to 1 per cent—and were inoculated into dogs in different amounts. Hence, for purposes of comparison, it seemed best to reduce the dose of each vaccine to an equivalent amount of 1 per cent emulsion. Thus, dogs of group A, given 5 cc of 20 per cent vaccine, are said to have received 100 cc of 1 per cent vaccine. On this basis, among dogs of groups A, B, and C receiving vaccine subcutaneously,

group C, given irradiated vaccine in a dose equivalent to 150 cc., suffered only 9.1 per cent mortality, in contrast to 46 per cent by group B, given a similar equivalent dose of chloroformized vaccine, and 77.3 per cent by group A, given phenolized vaccine. Again, among groups vaccinated intraperitoneally, group D, given phenolized vaccine in a dose equivalent to 500 cc., suffered a mortality of 22.2 per cent and group E, with a similar dose of chloroformized vaccine, 18.5 per cent as contrasted with 10 per cent by group F, given only 30 to 40 cc. of 1 per cent irradiated vaccine, or less than one tenth of that given to groups D and E, and 3.2 per cent by group G, given 150 to 300 cc. of irradiated vaccine.

TABLE VII

Relative Amounts of Irradiated Phenolized and Chloroformized Vaccines Required to Immunize Dogs

Group	Equivalent amount of 1 per cent vaccine	Total dead/Total for test	Dead
	cc.		per cent
A. Vaccinated subcutaneously—phenolized 20 per cent, 5 cc.	100	41/53	77.3
B. Vaccinated subcutaneously—chloroformized 33 per cent 5 cc.	150	23/50	46.0
C. Vaccinated subcutaneously—irradiated 5 per cent, concentrated 6 times, 5 cc.	150	1/11	9.1
D. Vaccinated intraperitoneally—phenolized 20 per cent, 25 cc.	500	2/9	22.2
E. Vaccinated intraperitoneally—chloroformized 33 per cent, 10–25 cc.	330–825	5/27	18.5
F. Vaccinated intraperitoneally—irradiated 1 per cent 30–40 cc.	30–40	3/30	10.0
G. Vaccinated intraperitoneally—irradiated 5 per cent, concentrated 6 times, 5–10 cc.	150–300	1/31	3.2

Actually these comparisons should be made not in terms of equivalent dose of 1 per cent vaccine but number of mouse lethal doses contained in the original vaccine material (1). This information is not obtainable for the commercial canine vaccines but for the irradiated preparations we find from the present tests that 10 cc. of the 1 per cent vaccine contains about 3×10^7 mouse doses and that this does not give the best immunity according to our rigid test, 5×10^7 or 10^8 doses, however, immunize dogs well.

The question of whether vaccine is more effective if given intraperitoneally rather than subcutaneously has not yet been definitely answered. Certainly in mice, the intraperitoneal is the route of choice (1). In dogs, however, the evidence is less convincing. Johnson and Leach (6) interpret their tests as showing the subcutaneous route to be more effective than the intraperitoneal,

to vaccinated animals was 92.2 per cent. Similarly, the mortality of fifty-three dogs given commercial phenolized vaccine according to directions was 77.3 per cent and that of fifty dogs given commercial chloroformized vaccine, 46.0 per cent. In contrast, of thirty-eight dogs given 20 to 40 cc of irradiated vaccine intraperitoneally, 13.1 per cent succumbed, and of thirty-one given 5 to 10 cc of irradiated vaccine, concentrated, only one succumbed to the test inoculation of virulent virus. Clearly the course of vaccination with irradiated vaccine appears far superior to that of vaccination with commercial vaccines.

From the figures of Table VI alone, however, one would rightly question whether the superior results with irradiated vaccine were due only to the quality of the material, or to the greater amounts employed and to the routes of inoculation. It is our belief that all three factors contribute to the potency of the

TABLE VI

Comparison of Commercial (Phenolized, Chloroformized) and Irradiated Canine Antirabies Vaccines

Groups		Total dead/ Total tested	Dead <i>per cent</i>
A	Controls Fixed virus, dilution 1:50 to 1:10,000, street virus, dilution 1:20	130/141	92.2
B	Vaccinated. Phenolized vaccine, 5 cc, subcutaneously	41/53	77.3
C	Vaccinated. Chloroformized vaccine, 5 cc, subcutaneously	23/50	46.0
D	Vaccinated. Irradiated brain tissue supernatant		
	1 20-40 cc, intraperitoneally	5/38	13.1
	2 5-10 cc concentrated, intraperitoneally	1/31	3.2

irradiated vaccine and the following reasons are emphasized. That irradiated vaccine given intraperitoneally is superior to equal quantities of chloroformized vaccine given by the same route is indicated by the quantitative results of three experiments (1) in which one part of a single pool of virus material was inactivated by chloroform and another part by irradiation. Each inactivated preparation was then given to mice as vaccine and in each instance the irradiated sample proved equal in immunizing potency or superior to the chloroform-treated sample. In further tests, the irradiated vaccine was compared with commercial canine vaccines for ability to immunize dogs. The results shown in Table VII, though not critical, confirm the findings in mice. The vaccines contained widely different amounts of brain-virus—33 per cent to 1 per cent—and were inoculated into dogs in different amounts. Hence, for purposes of comparison, it seemed best to reduce the dose of each vaccine to an equivalent amount of 1 per cent emulsion. Thus, dogs of group A, given 5 cc of 20 per cent vaccine, are said to have received 100 cc of 1 per cent vaccine. On this basis, among dogs of groups A, B, and C receiving vaccine subcutaneously,

ADSORPTION OF INFLUENZA HEMAGGLUTININS AND VIRUS BY RED BLOOD CELLS

By G. K. HIRST M.D.

(From the Laboratories of the International Health Division of The Rockefeller
Foundation, New York)

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In a recent paper on the agglutination of chicken red blood cells by suspensions of influenza virus (1), the majority of the experiments dealt with the agglutination inhibiting properties of immune sera, and correlations were drawn between measurements of influenza antibodies done *in vitro* and those obtained by protection tests done in mice. In the present paper are reported the results of experimentation on the agglutination reaction itself.

It has previously been shown that a high percentage of the infective agent was removed from an influenza virus suspension when washed chicken cells were added and centrifuged out at low speed (2). The adsorption of influenza virus on chicken red cells was confirmed by the work of McClelland and Hare (3) who showed in addition that the adsorbed virus could be detected on the sedimented cells. Further work on the hemagglutinins present in influenza virus suspensions and on their adsorption on red cells is reported in the present communication.

Methods

Preparation of Virus Suspensions—The two viruses used in this work were the mouse virulent PR8 strain of influenza A virus (4) and the Lee strain of influenza B virus (5). Suspensions of both strains were prepared from allantoic fluid by a technique which has been previously described (6). Briefly the method consisted of inoculating the allantoic sac of 11-day old white Leghorn embryos with 0.1 cc. of a 10^{-3} dilution of infected allantoic fluid. The eggs were incubated at 37°C for 48 hours, and the allantoic fluid was removed without contamination with embryonic red cells. The cellular debris was removed from the fluid by low speed centrifugation. Large pools of allantoic fluid containing each virus were made at one time. The pools were distributed in small amounts into lusteroid tubes, which were stored at -72°C until used. All of the work reported here was done with fluid from these pools.

Agglutination Titrations—All estimations of agglutination titer were done in the same manner. Twofold dilutions of the virus suspension were made in saline. The test tubes used had an internal diameter of 0.8 cm. and a length of 7 cm. To 1 cc. of each dilution was added 1 cc. of a 1.5 per cent suspension of chicken red cells. The cells were added with an automatic pipetting machine which delivered them with such force that sufficient mixing took place at once. The degree of agglutination was read after the suspensions stood 75 minutes at room temperature.

whereas our data, though not based on tests especially planned to decide this question, suggest that there is little difference between the two routes but that the intraperitoneal is slightly preferable

COMMENT AND SUMMARY

Our studies on rabies vaccines thus far have led us to the view that in order to develop and test vaccines, quantitative methods are necessary, and that such quantitative methods may be exploited to greatest advantage by using mice, preferably W-Swiss, as the test animal. Dogs, due to their variability and susceptibility to intercurrent infections when kept under experimental conditions, are useful chiefly to check whether or not a vaccine produces a high grade of immunity, they remain of limited value in testing the comparative potencies of weak vaccines. A second point is that the Pasteur strain of virus has proved as potent as any tested for the preparation of vaccines.¹ Another point is that virus material for preparing vaccines must titre at least 330,000 mouse doses per cc to be effective. This requirement has eliminated all culture vaccines thus far reported, with the possible exception of Plotz's (7) and leaves virus-containing brain tissue as the sole potent source of vaccine.

In summary, we believe that a single injection of non-virulent irradiated vaccine, prepared as herein described, immunizes mice and dogs effectively against a subsequent test inoculation of virulent rabies virus and does so to a greater degree than do other vaccines now obtainable. It is easily and quickly prepared, keeps well, and has a low nitrogen content.

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¹ Casals, in unpublished studies, has found the Pasteur strain to be somewhat superior as an antigen to several other recently isolated strains.

the usual way except that the virus was diluted in twofold steps. The 50 per cent mortality titer was calculated from the deaths which occurred within the first 10 days after inoculation

EXPERIMENTAL

Adsorption and Elution of Influenza Hemagglutinins by Chicken Red Blood Cells—50 cc. of a suspension of Lee virus and 50 cc. of a 3 per cent suspension

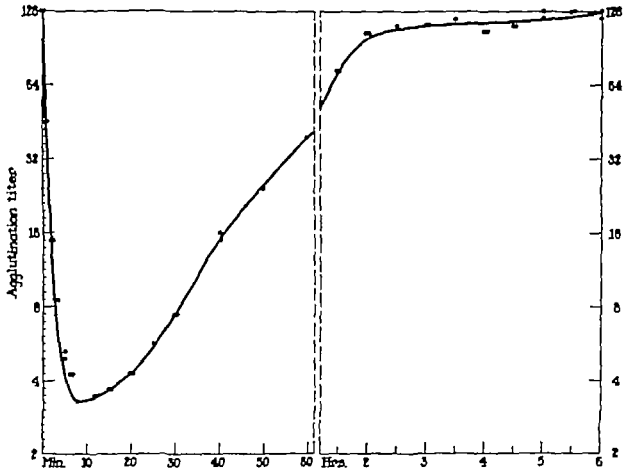


FIG 1 Supernatant agglutination titers on a suspension of Lee allantoic fluid after the addition of chicken red blood cells. For this experiment the final dilution of virus preparation was 1:2, the final concentration of red cells was 1.5 per cent, and the adsorption was carried out at 23°C.

of chicken red cells were brought to room temperature and were then thoroughly mixed. 5 cc. samples were removed at intervals, the suspension being well mixed each time. The red cells in the samples were quickly sedimented at low centrifugal speed and the supernatant fluids were pipetted off the cells. After the collection of all the samples the supernatant fluids were tested in duplicate for agglutination titer.

The results of these titrations are plotted in Fig. 1 and show that the influenza hemagglutinin was rapidly adsorbed from suspension. 9 minutes after the addition of red cells 97 per cent of the agglutinin present had been adsorbed.

The method of estimating the degree of agglutination and of calculating the agglutination titer was changed from that formerly used (6). Previously we measured the amount of agglutination in a given tube by comparing the density of the suspended cells in the supernatant portion of the fluid column with the density of standard red cell suspensions. In the present work the densities of the supernatant red cell suspensions were estimated by measuring the amount of light transmitted through a portion of the supernatant by means of a photoelectric densitometer. The details of construction of this optical densitometer will be given in a later report (7). The galvanometer readings were converted into values expressing the percentage of cells added to a tube, which remained in suspension after 75 minutes. The end point of a titration was taken to be the dilution of virus suspension where 50 per cent of the original cells added had sedimented to the bottom during the incubation period. Thus if a 1.5 per cent suspension of cells was added to a titration (1 cc. of virus dilution plus 1 cc. of cell suspension), the end point was in a tube with a supernatant cell density equal to that of a 0.37 per cent suspension of red cells. Usually, however, the 50 per cent sedimentation point fell between two dilutions, and it was necessary to calculate the titer by calculation from the two values obtained from dilutions on either side of the end point. This calculation was based on the fact that in an agglutination titration when the percentage of cells sedimented was compared with the dilution of virus suspension causing the agglutination, the curve for sedimentation was linear over a wide range when the dilution of virus was plotted on a logarithmic scale (7).

For example, in an agglutination titration 42 per cent of the cells in the 1:128 virus dilution remained suspended at 75 minutes, while in the 1:256 dilution 56 per cent of the cells did not completely sediment. The 50 per cent sedimentation point was calculated as $\frac{50 - 42}{56 - 42}$ or $\frac{8}{14}$ of the distance between the two dilutions. Since the scale of dilution was logarithmic, this distance was $\frac{8}{14} \times \log 2$ or $0.57 \times 0.301 = 0.172$. $\log 128 = 2.107 + 0.172 = 2.279$, and antilog 2.279 = 190, which was the final titer. All titers are expressed as the reciprocal of the final dilution of the original virus suspension.

The use of a photoelectric densitometer for these readings has increased the accuracy of determination of end points over the visual method formerly employed. In general, duplicate determinations of agglutination titer checked within less than ± 10 per cent of the estimated value, although occasional duplicate titrations showed greater disparity than this.

Chicken Red Cells—All of the red cells used were obtained from chickens in a local slaughterhouse. After the cervical vessels were severed the chicken blood was collected in one volume of 2 per cent sodium citrate. The cells were washed three times in physiological saline and were centrifuged at 1000 R.P.M. for 4 minutes after the final washing. The packed cells were stored at 4°C and were diluted before use in saline. 1.5 per cent suspensions (by packed volume) were used for agglutination titrations. The suspensions, first made up by volume, were changed to a standard density (as measured on the optical densitometer) by the addition of small amounts of cells or saline.

Virus Titrations in Mice—Titrations of virus suspensions in mice were done in

virus. Equal volumes of 0.75, 1.5, and 3.0 per cent red cell suspensions were added to PR8 allantoic fluid, and the supernatant titers were determined as before. Regardless of the concentration of cells used the time of maximum adsorption was the same, about 1 hour. With large concentrations of cells the maximum adsorption was more complete than with small concentrations, but correspondingly the subsequent elution of agglutinin was less complete in 6 hours when more cells were used. This experiment was carried out at room temperature (23°C).

Adsorption of the PR8 Virus Hemagglutinin at Various Temperatures—In order to test the effect of temperature on the adsorption-elution phenomenon a preparation of PR8 virus was used, and the reaction was carried out at 4°, 27°, and 37°C. Equal volumes of virus preparation and a 3 per cent suspension of red cells were both brought to corresponding temperatures before mixing together. Samples were taken as before, and the supernatants were all tested in duplicate for agglutination titer at room temperature. The results are shown graphically in Fig. 3.

To evaluate the results of this experiment it is easiest to consider the different phases of the adsorption and elution separately. The speed of the initial adsorption in the first minute was practically the same at the different temperatures. The difference in speed of adsorption at 4°C. and at 27°C. was very small for as long as 30 minutes. The most striking variation occurred in the time of maximum adsorption of the agglutinin. At 4°C. this was approximately 5 hours, at 27°C. the maximum occurred at 25 minutes, and at 37°C. this point fell between 3 and 5 minutes. Likewise when the degree of adsorption which occurred at the maximal points is considered, the percentage of total agglutinin adsorbed at 4°C. was found to be 99.5 per cent, at 27°C. it was 98.8 per cent while at 37°C. only 87 per cent was adsorbed. In the elution phase of the experiment the differences found are also marked. At 4°C. less than 1 per cent of the agglutinin adsorbed had eluted in 18 hours. At 27°C. the elution was much more rapid but still very incomplete at 6 hours' time, only 25 per cent of the agglutinin having returned to the supernatant. The elution at 37°C. was much more rapid and was very nearly complete at 6 hours' time. A change in temperature from 4°C. to 37°C. greatly speeds up the elution phase of the reaction, and the curve obtained with PR8 virus at 37°C. resembles that found with Lee virus at 23°C. (Fig. 1).

Effect of Adsorption and Elution of Hemagglutinins on the Red Cells—When chicken red cells are agglutinated by preparations of influenza virus, the clumps of red cells formed are very fragile and are easily broken up by gently shaking the tube which contains them. When the clumps are broken up so that the cells form a well dispersed suspension again, frequently the cells will agglutinate just as they did before and under suitable conditions such reagglutination may be demonstrated many times.

In the previous experiments on the adsorption of PR8 virus hemagglutinins at different temperatures (Fig 3) some of the adsorbing cells were removed at various times for the testing of their agglutinability at different stages in the adsorption. For this purpose 2 cc of the mixed suspension were used for each test. The cells and supernatant were thoroughly mixed with a pipette until

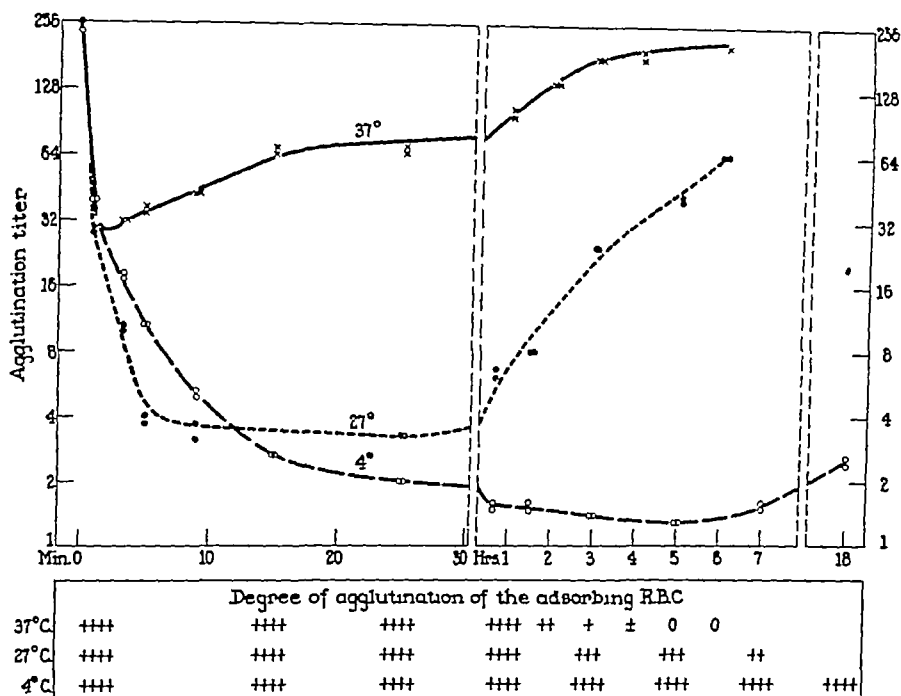


FIG 3 Supernatant agglutination titers on suspensions of the PR8 virus strain. The adsorptions were carried out at 4°, 27°, and 37°C. The final dilution of virus suspensions was 1/2, and the concentration of cells was 1.5 per cent. At the bottom is indicated the degree of agglutinability of the adsorbing cells when removed from the suspension at various times. +++ indicates the maximum degree of agglutination usually seen, and zero indicates no agglutination.

the suspension was even and showed no clumps, and then was put into a regular agglutination test tube and the degree of agglutination (or reagglutination) was read in 75 minutes. The results of these tests are recorded on the bottom of Fig 3. Four plus indicates the maximum degree of agglutination usually seen under any conditions, and lesser degrees of aggregation are recorded as three plus to plus-minus. Zero indicates that the cells did not agglutinate at all. When the adsorption was carried out at 4°C, the cells retained their full capacity to agglutinate for at least 18 hours, and, as has been pointed out previously,

the cells retained almost all of their adsorbed agglutinin for this time. When the adsorption was carried out at 27°C. the agglutinability of the cells decreased as a significant amount of agglutinin was eluted, but aggregation still occurred after 7 hours' exposure to the virus. At 37°C. the ability of the cells to agglutinate disappeared at 5 hours. Cells removed from the 37°C. preparation also failed to agglutinate when suspended in fresh virus preparations of either the PR8 or the Lee strain.

Red cells which were mixed with Lee allantoic fluid at room temperature also showed with time a similar decrease in capacity to agglutinate. After 4 to 5 hours' exposure to the virus suspension, the cells failed to reagglutinate at all, either in saline or in fresh virus suspensions of the same or heterologous strains.

While the foregoing tests showed that cells which were exposed for long periods to virus suspensions lost their agglutinability, it was decided to see if such inagglutinable cells were capable of adsorbing freshly added virus agglutinin. A 1.5 per cent suspension of red cells was added to a preparation of the Lee strain of influenza virus, and after 4 hours the cells were removed and added to a fresh suspension of virus prepared from the same strain, and the supernatant agglutinin titers were measured as before. Fresh red cells were added to more of the same virus suspension as a control on the adsorption. The results of these two adsorption tests are shown in Fig. 4. The control adsorption with fresh red cells gave the usual result, while the previously treated cells failed to adsorb a significant amount of agglutinin from the new virus suspension. The failure of these treated cells to adsorb any agglutinins on second exposure was not due to the fact that the cells were "saturated" with agglutinin, because in the first exposure the agglutinating agent had been almost completely eluted from the cells.

In a similar manner it has been shown that cells exposed to PR8 virus for 4 to 6 hours also fail to adsorb agglutinins from fresh suspensions of the same strain. In addition, it was found that red cells exposed to the PR8 strain failed to adsorb agglutinins from suspensions of Lee virus and vice versa.

These experiments show that the exposure of chicken red cells to either strain of virus alters the cell in such a way that it is no longer agglutinable and no longer has the power to adsorb influenza hemagglutinins. It also seems likely that a certain amount of agglutinin must be fixed to the cell in order for agglutination to occur. The loss of agglutinability and the elution of the agglutinin are parallel phenomena.

Activity of the Hemagglutinin Following Its Adsorption and Elution from Chicken Red Cells—The following experiment was performed to determine whether the agglutinin which has once been adsorbed on red cells and subsequently eluted is still capable of further similar activity. 1.5 cc. of packed red cells was added to 100 cc. of Lee allantoic fluid. Samples were removed periodically, the cells sedimented, and the supernatant fluids removed. After 3 hours

all of the cells were removed from the remaining virus suspension, and fresh, packed cells were added in sufficient quantity so that the final concentration was 1.5 per cent. Samples were removed for another 3 hours, the second lot of cells was removed, and a third time fresh red cells were added. This suspension was sampled for a final 3 hours. All of the supernatant fluids were tested in duplicate for agglutination titer at one time. The results of this experiment

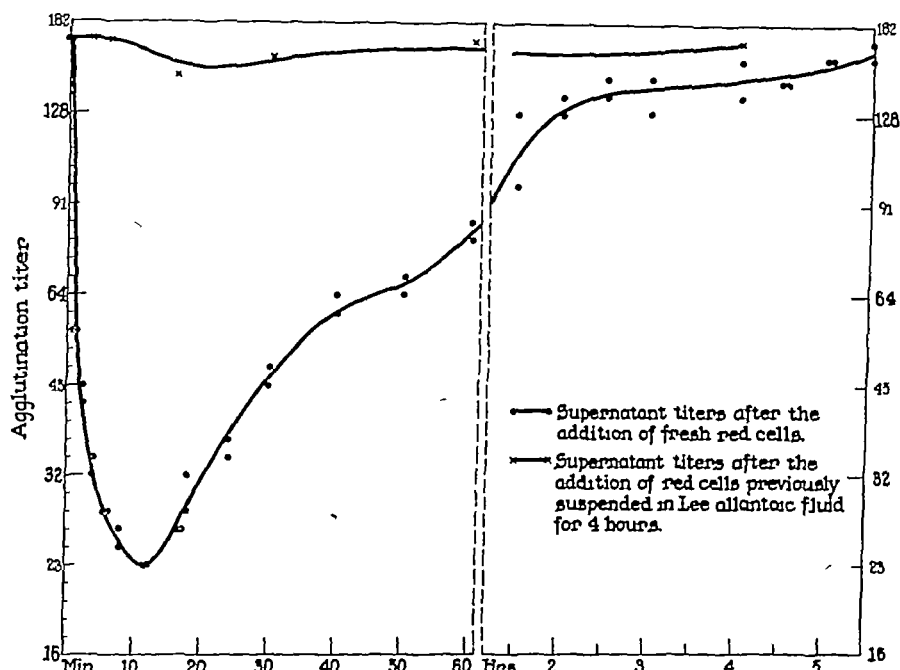


FIG 4 Supernatant agglutination titers on suspensions of the Lee strain of influenza virus after the addition of fresh red cells and cells which had previously been exposed for 4 hours to Lee virus. The final concentration of virus was 1.2 in each case, while that of the cells was 0.75 per cent. The tests were carried out at 24°C.

are shown in Fig 5, and they clearly indicate that the activity of the agglutinin is not exhausted by the process of adsorption and elution, and except in a minor degree no activity is lost in the process.

Stability of the Red Cell Factor—Stroma was prepared from chicken red cells by homogenizing a concentrated suspension in a Waring mixer. The sediment from the fragmented cells was washed repeatedly in distilled water and in saline until no red color remained. When this stroma was added to virus suspension the hemagglutinin was rapidly adsorbed and elution began to occur after 10 to 15 minutes when the Lee strain of virus was used. The adsorption curves were quite similar to those obtained with the use of intact cells.

Chicken red blood cells were heated to 65°C for $\frac{1}{2}$ hour. At this temperature the cells were completely lysed, and the stroma was used for adsorption tests. The adsorption curves were similar to those obtained with intact cells. When the cells were heated at 100°C for 5 minutes, adsorption and elution still occurred with the stroma, but the elution of the agent was much less marked than with the use of intact cells. In all of the experiments with stroma, heated

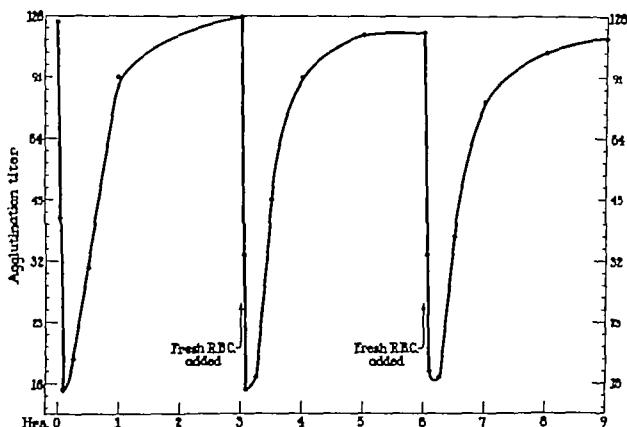


FIG 5 Supernatant agglutination titers on a suspension of the Lee strain of influenza virus, to which fresh red blood cells were added three times at three hourly intervals. The final concentration of the virus suspension was approximately 1:1 throughout, while the concentration of cells was maintained at 1.5 per cent. The test was carried out at 24°C.

or unheated, a much larger amount of substance was necessary to produce a good adsorptive effect than if intact cells were used.

Stability of the Virus Hemagglutinins—In order to determine the degree of heat stability of the influenza hemagglutinins, suspensions of PR8 and Lee virus were heated at 50°C, 55°C, and 60°C for 1 hour. Samples were removed periodically and tested for agglutination titer. Each sample was also given intranasally in full concentration to six mice. The effect of heating on the parallel *in vitro* and *in vivo* tests is given in Table I. A temperature of 50°C for 1 hour does not detectably affect the agglutination titer. At 55°C about half the agglutinating activity is lost from each suspension in 1 hour. At 60°C the inactivation is greatly enhanced. This is an average result, but the exact

degree of inactivation at a given temperature varies with different lots of virus. The loss of infectivity does not parallel the drop in agglutination titer.

TABLE I
Inactivation of Influenza Virus Hemagglutinins and Infectivity by Heat

Strain	Temperature	Agglutination titer						
		Length of time at given temperature (min)						
		0	1	5	15	30	45	60
PR8 (A)	°C							
	50	447*	479*	447*	479*	447*	479*	479*
	55	479*	338*	362*	315	223	194	223
Lee (B)	60	447*	169	9	6	5	3	<2
	50	194*	169*	194*	194*	182*	208*	208*
	55	208*	147*	128	128	104	97	91
	60	194*	56	<2	<2	<2	<2	<2

All samples were inoculated intranasally in mice, 0.05 cc of undiluted material.

* Indicates those groups of mice in which death or lung lesions occurred as a result of the inoculation.

TABLE II
Inactivation of Influenza Virus Hemagglutinins and Infectivity by Formaldehyde

Strain	Concentration of formalin	Agglutination titer			
		Length of time of exposure to formalin			
		30 min	1 day	3 days	8 days
PR8 (A)	<i>per cent</i>				
	None	256*	239*	256*	208*
	0.1	294*	138	128	91
	0.5	223	147	52	<2
	1.0	194	97	<2	<2
Lee (B)	None	147*	128*	128*	128*
	0.1	128*	138	128	128
	0.5	138	138	104	52
	1.0	120	104	34	<2

All samples were inoculated intranasally in mice, 0.05 cc of undiluted material.

* Indicates those groups of mice in which death or lung lesions occurred as a result of the inoculation.

A preparation of PR8 virus was heated at 55°C for 30 minutes. No infectivity was then detectable, and about half of the agglutinating power was lost. Red cells were added to this suspension, and the adsorption of the remaining agglutinin was measured. Adsorption and elution occurred in a manner similar to that obtained with untreated virus suspensions.

To find out the effect of formaldehyde on the hemagglutinins, again suspensions of the PR8 and Lee strains were used. Formalin was added to aliquots of each virus to a final concentration of 0.1, 0.5 and 1.0 per cent. After $\frac{1}{2}$ hour the various suspensions were tested for agglutination titer. They were retested at 1, 3 and 8 days, the suspensions being stored at 4°C. Parallel tests for in

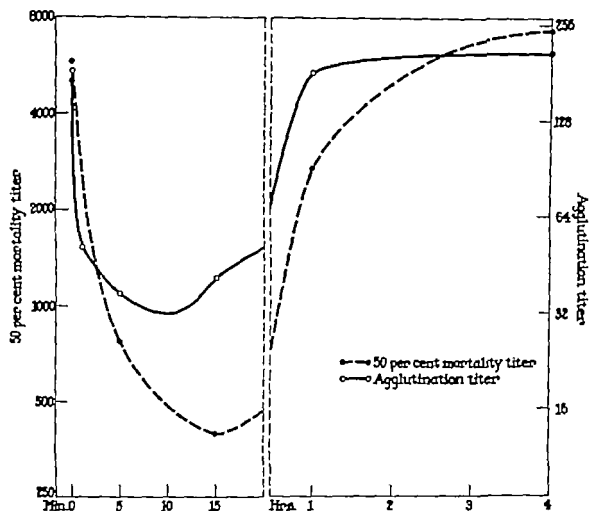


FIG. 6 Supernatant agglutination titers and 50 per cent mouse mortality titers on a suspension of the Lee strain of influenza virus, to which chicken red cells were added. The final concentration of the virus suspension for the test was 1:2 and of the cells 0.75 per cent.

fectivity were run by inoculating mice with 0.05 cc. of the undiluted material. The results are recorded in Table II from which it can be seen that formaldehyde has a slow inactivating effect on the agglutinin and a rapid and marked effect on the infectivity.

Formalin was added to suspensions of PR8 virus in such amount that after the addition of an equal volume of 1.5 per cent red cells the concentration was 0.1, 0.5, 1.0, and 2.0 per cent. Titrations were run on the supernatants up to 4 hours. Formalin concentrations of 0.1 and 0.5 per cent appreciably retarded the adsorption and elution of agglutinin. In the presence of 1.0 and 2.0 per

cent formalin, however, the agglutinin was rapidly adsorbed and there was no evidence of subsequent elution

Correlation between the Adsorption and Elution of Virus Hemagglutinins and Infectious Activity—While it has already been shown that chicken red cells adsorb virus infective activity from a suspension (2, 3), it has not been shown whether or not the infective particle is subsequently eluted from the cells. For this purpose a preparation of Lee virus and an equal volume of 1.5 per cent red cells were mixed. Samples were withdrawn at various intervals, from which the red cells were removed and the supernatant fluids were tested for agglutination titer *in vitro* and for mortality titer in mice. The two series of titers obtained are shown in Fig. 6. The agglutination titrations gave the usual type of curve with maximum adsorption at about 10 minutes, when approximately 80 per cent of the agglutinin had been removed. The 50 per cent mortality titers showed a maximum adsorption at 15 minutes when 95 per cent of the original infectivity was removed. In 4 hours' time both the agglutination titer and the mouse mortality titer of the supernatant fluid returned to their original values. Both forms of activity began to elute at about the same time. This result adds one further bit of evidence in favor of the view that the infective activity and the agglutinating activity in a virus suspension are very closely associated and may constitute parts of the same particle.

DISCUSSION

The foregoing data are much too meager to permit any definite conclusions to be drawn regarding the mechanism of agglutination of chicken red cells by suspensions of influenza virus. The facts do, however, suggest two analogies which may be of some assistance in planning a further attack on the problem.

The adsorption of bacteriophage onto the susceptible bacterial cell is very similar to the reaction we have described. In this case the bacteriophage particle is believed to attach to specific receptor points on the bacterial surface, and in instances in which the receptors have been isolated they have proved to be polysaccharides (8). Cells which do not possess the receptor points do not adsorb the phage. With bacterial viruses, however, the adsorption of the phage particle is followed by multiplication and then release of new particles. Here the analogy ends since there is no evidence so far of the ability of influenza virus to multiply in the presence of avian red cells.

The curves of adsorption and elution of influenza hemagglutinins suggest that the interaction of hemagglutinin and virus occurs in two phases: first, a combination (rapid even at low temperatures) and second, some alteration of the cells accompanied by a separation of the modified cells and the agglutinin. The latter phase appears to be considerably retarded at low temperatures. The modification of the cells in the second phase renders them incapable of combining with more agglutinin, while the released agglutinin is apparently unchanged.

The analogy of this reaction with the interaction of enzymes and substrates seems worthy of note. It is the generally accepted view that the first stage in enzyme action—at least in most cases—is a combination of enzyme and substrate. Then the substrate is chemically changed, whereupon the enzyme and the altered substrate dissociate, and the enzyme is free to adsorb and alter more substrate. It is clear that if one were able to make a measurement of the amount of free enzyme present during this process, the amount of free enzyme should be low in the initial stages after combination had occurred in the presence of an excess of substrate. In the later stages of enzyme action when the substrate has been largely used up, free enzyme should appear again in quantity approaching the initial concentration, since there is insufficient substrate to combine with all the enzyme present.

In this analogy the agglutinin corresponds to the enzyme, which is not used up, while the substrate corresponds to the substance at the receptor point on the red cell, which is destroyed during the process of agglutination, rendering the cell incapable of further adsorption. The sensitivity of the hemagglutinin to heat and to formalin is consistent with a substance of protein nature. The cellular substrate, on the other hand, is very resistant to heat, and this stability suggests that it may be a non-protein substance.

The obvious approach to the problem of the nature of this agglutination reaction is to try to isolate from the red cell the receptor substance and then to see what effect the virus suspensions have on it.

The simplest explanation of the actual aggregation of cells in the presence of the agglutinin is to view the latter as forming a bond between the cells. If the agglutinating particles were bi- or trivalent and there were multiple receptor points on the red cells, one could visualize how a network or clump of cells could be built up. The main evidence for this view is the fact that the agglutinability of the cells and the amount of virus hemagglutinin adsorbed are parallel phenomena.

Since the infective agent in a virus suspension was adsorbed and eluted in a manner similar to that of the hemagglutinin, it is clear that there is a close association between these two types of activity. Whether or not these two agents occur on the same particle or on different particles, and whether or not the two forms of activity are completely separable is not yet known. So far the agglutinin may be obtained without virus activity, but we have not yet been able to inactivate the agglutinin without also inactivating the virus.

It seems likely that the agglutination of red cells by influenza hemagglutinins may have some counterpart in natural infection of susceptible cells with the virus. While there is no evidence at all concerning this problem at present, it may be that susceptible cells possess a surface receptor similar to that of the red cell and that in infection this substance is altered by the hemagglutinin, thus injuring the cell membrane and providing a point of attack for the infective particle.

SUMMARY

A number of experiments were performed on the adsorption of influenza hemagglutinins on chicken red blood cells, from which the following conclusions were drawn —

1 When chicken red blood cells and preparations of influenza viruses were mixed together, the influenza hemagglutinins present were rapidly adsorbed onto the cells. After varying lengths of time, dependent on the conditions of the experiment, the adsorbed hemagglutinins began to elute from the cells. With the Lee strain at 23°C and the PR8 strain at 37°C almost all of the adsorbed agglutinin was released in 4 to 6 hours.

2 When the number of red cells used for adsorption was increased, the speed and degree of adsorption of the hemagglutinins increased. The time of maximum adsorption of hemagglutinins was the same, regardless of red cell concentration, and with the larger amounts of red cells the speed and degree of elution was decreased.

3 When adsorption of PR8 virus agglutinins was carried out at 4°C the adsorption was rapid and nearly complete. When the reaction was carried out at higher temperatures (27° and 37°C), the adsorption was equally rapid but was progressively less complete with rise in temperature. At 4°C the maximum adsorption was not reached for 5 hours, at 27°C it was reached in 25 minutes, and at 37°C the greatest degree of adsorption was attained between 3 and 5 minutes. The amount of elution observed at 4°C at 18 hours was negligible, but the degree of elution increased with temperature so that at 37°C almost all of the adsorbed agglutinin was released in 6 hours' time.

4 Red cells which had adsorbed and then fully eluted the agglutinin were not capable of adsorbing a detectable amount of fresh agglutinin. In addition, such cells would no longer agglutinate even though exposed to fresh virus suspensions.

5 The hemagglutinin of influenza B virus was capable of being adsorbed on and eluted from several successive lots of chicken red cells without appreciable loss of agglutinating activity.

6 The hemagglutinins of the PR8 and Lee strains were rapidly inactivated at 60°C. The presence of active virus was not necessary for the occurrence of the adsorption-elution reaction on chicken red cells.

7 The activity of the portion of the red cells responsible for the adsorption of the hemagglutinins persisted, though in reduced amount, even after heating for 5 minutes at 100°C. Hemagglutinins were adsorbed and eluted from red cell stroma.

8 The infective agent in influenza virus suspensions was adsorbed by chicken red cells simultaneously with the adsorption of hemagglutinins. 95 per cent of the infective agent was removed from suspension by the red cells after contact for 15 minutes. From then on the infective agent was gradually released from

the red cells After 4 hours the 50 per cent mortality titer of the supernatant fluid was as high as at the beginning of the experiment.

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THE MANUFACTURE OF ANTIBODIES IN VITRO

BY LINUS PAULING PH.D., AND DAN H. CAMPBELL, PH.D

(From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California)

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Two years ago there was published (1) a theory of the structure and process of formation of antibodies which had been developed from the available information about intramolecular and intermolecular forces. The prediction was made from this theory that antibodies could be manufactured *in vitro* from suitable protein molecules by use of an appropriate general method, which was described as follows—

‘An interesting possible method of producing antibodies from serum or globulin solution outside of the animal is suggested by the theory. The globulin would be treated with a denaturing agent or condition sufficiently strong to cause the chain ends to uncoil, after which this agent or condition would be removed slowly while antigen or hapten is present in the solution in considerable concentration. The chain ends would then coil up to assume the configurations stable under these conditions which would be configurations complementary to those of the antigen or hapten.’

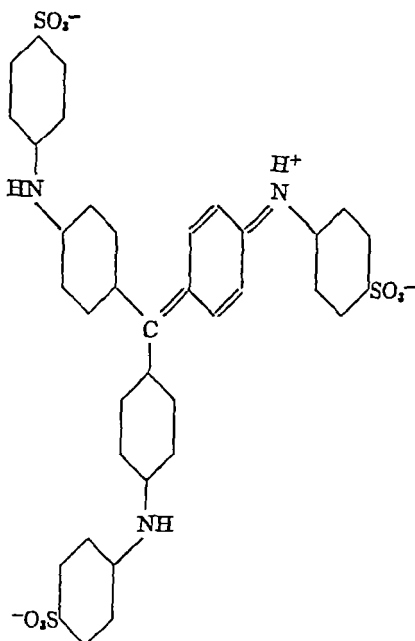
We have during the past two years carried out experiments along these lines, and have succeeded in endowing normal serum globulin with the properties of a specific antibody, in other words, we would seem to have converted normal globulin into antibody. Similar but less satisfactory results have been obtained also with other serum proteins. Our experiments are described in the following sections of this paper^{1 2}

Orienting Experiments with Methyl Blue as Antigen

Many orienting experiments were carried out with the dye methyl blue as antigen. This dye as obtained commercially is a mixture of the *p*-trisulfonated and disulfonated pararosanilines, with formulas such as

¹ A brief statement of the results of these experiments has been published Pauling L. and Campbell, D H., *Science* 1942, 95, 440

² We acknowledge with appreciation the financial support of this work by the Rockefeller Foundation. We are also indebted to Dr. David Pressman and Mr. Carol Ikeda of these Laboratories for help with the experiments, and to Dr. W. Goebel of The Rockefeller Institute for Medical Research, Dr. E. J. Cohn of Harvard Medical School, and Dr. J. D. Porsche of Armour and Co. for providing us with materials.



It was selected because of its high solubility in water, its intense color (giving increased accuracy to colorimetric analyses), its dialyzability, and the presence in the molecule of strong haptenic groups. The protein used for most of the preliminary experiments was a γ -globulin fraction obtained by essentially the methods employed by Hewitt (2) and by Cohn and collaborators (3) in their studies of serum proteins. It was the protein fraction from fresh beef serum which is insoluble at 33 per cent saturation with ammonium sulfate. The material was reprecipitated once, washed with salt solution, and then dialyzed free of ammonium sulfate at pH 7.5 and 4°C. The resulting solution was then stored in the refrigerator until used.

Of the five methods of denaturation used in the preliminary investigations, treatment with sodium hydroxide solutions gave the most promising results. A typical experiment was carried out as follows —

Enough dye was added to a 2.0 per cent protein solution to give a dye concentration of 0.1 per cent and enough 0.1 N NaOH to raise the pH to approximately 11.0. After the mixture had stood for 15 to 30 minutes, a 10.0 ml sample was removed, placed in a cellophane bag, and dialyzed at 4°C against a stream of phosphate buffer of pH 7.5. In this way the protein molecules, which had been subjected to the denaturing action of the alkali, were given the opportunity of changing slowly to configurations stable in neutral solution and in the presence of the antigen. The renaturing process took place over a period of several hours, during which the pH of the protein-antigen solution changed slowly from 11.0 to 7.5. At this point a small amount of protein-dye precipitate had appeared. On continued dialysis, with loss of dye, the amount of

precipitate increased. At the end of 24 hours only a small amount of dye remained in solution, the rest having been precipitated or dialyzed through the cellophane. The dialysis was continued for 72 hours at the end of which only a trace of dye remained in solution.

Various control experiments were made such as the following. A 10 ml. sample of the dye-protein alkali solution which had stood at pH 11.0 for 15 to 30 minutes was brought to pH 7.5 very rapidly by addition of 0.1 N HCl solution and was then dialyzed as above. A second control experiment was made with no addition of alkali; the dye-protein solution at pH 7.5 was dialyzed. In both of the controls only a trace of dye remained in solution after dialysis.

The precipitates were dissolved in dilute NaOH solution and analyzed for dye colorimetrically and for protein by Kjeldahl nitrogen determination. A large amount of precipitate was formed both in the main experiment (with slow neutralization) and in the first control, however, the precipitate from the main experiment contained 80 μ g of dye per mg protein, and that from the first control only 6. Only a small amount of precipitate with 3 μ g of dye per mg protein was obtained in the second control. The precipitate from the main experiment was soluble in excess dye and in sodium *p*-sulfanilate solution (hapten) but not in other unrelated dyes or hapten solutions, such as sodium *p*-arsanilate solution. The control precipitates were in soluble or only slightly soluble under these conditions.

These facts indicate that the precipitate obtained by slow removal of the denaturing agent (hydroxide ion) is a specific antigen-antibody precipitate, that obtained in the first control being denatured protein and that obtained in small quantity in the second control being non-specific dye-protein complex.

Various methods were tried, without success, to separate the dye from the dye-protein precipitate and to obtain a specific precipitin for the dye. Methods used consisted of treating the dye-protein precipitate with acid and alkaline solutions, strong salt solution (20 per cent NaCl) and dialysis against solution of hapten (*p*-sulfanilate ion). The precipitate dissolved in relatively strong alkaline solutions and the dye could then be removed by dialysis but the resulting protein had lost its affinity for dye. The precipitate did not dissolve in acid or strong salt solutions nor was the dye removed by dialysis against these solutions. Some of the precipitate dissolved in 0.01 N hapten solution but the dye was not removed by dialysis against this solution. Our later experience with arsanilate indicates that this failure was due to the use of too low hapten concentration.

Other methods of denaturation gave varying results. Heat denaturation at various temperatures up to 80°C with slow cooling produced approximately the same effect as alkali denaturation. Acid treatment at pH 2.5 led to no alteration in affinity for dye. Although 3.5 N urea solutions produced considerable denaturation there was no evidence of increased affinity for dye. Surface denaturation brought about by long continued shaking of a 1 per cent protein solution in the presence of dye gave a precipitate containing approximately 200 μ g of dye per mg of protein. However, the precipitate formed

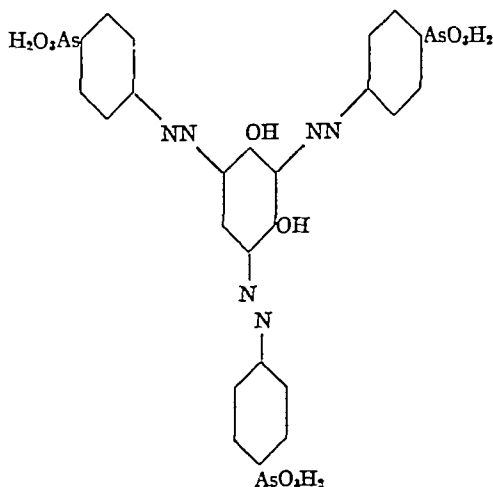
by this method was not soluble in excess dye or in hapten solution, and hence in this respect is not closely similar to an antigen-antibody precipitate. We think that surface denaturation can probably also be developed into a satisfactory method of *in vitro* manufacture of antibodies.

Although most of the investigations were carried out with the γ -globulin fraction, alkali denaturation tests were also made with other serum fractions obtained by salt precipitation. All of these fractions, including serum albumin, showed on treatment an increase in dye affinity, somewhat smaller than that shown by the γ -globulin fraction.

The Manufacture of Antibodies Homologous to the Azophenylarsonic Acid Group

In other experiments which led to the preparation of protein solutions with properties of specific antisera a different method of causing the change in configuration of the protein molecules was used, namely, increased thermal agitation at a temperature lower than that which leads to precipitation of denatured protein. At a temperature just below the "denaturation temperature" (at which precipitation occurs) the protein molecules would be expected to assume various configurations and to change more easily from one configuration to another than at room or body temperatures. It was accordingly expected that under these conditions the protein in the presence of antigen would change from its normally stable configuration to configurations stable in the presence of antigen molecules, namely, those complementary to active parts of the antigen molecules.

This method was carried out with another dye, 1,3-dihydroxy-2,4,6-tri(*p*-azophenylarsonic acid)benzene, which had been found (4) to give very good precipitates with hapten-homologous rabbit antiserum. This dye, which contains three azophenylarsonic acid groups attached to a resorcinol molecule, has the following formula —



It dialyzes through cellophane, and its color is intense enough to permit accurate colorimetric analyses to be made

The γ -globulin used in these experiments was prepared from fresh beef plasma by ethanol precipitation as described by Cohn and collaborators (5). At first the experiments were carried out with material prepared in our laboratory, and the later work was done with preparations kindly supplied through the courtesy of Dr. E. J. Cohn of Harvard Medical School and Dr. J. D. Porsche of Armour and Co. No significant difference in behavior was observed between the two preparations.

Preparation of the modified protein was carried out as follows —

A solution containing 0.01 per cent of dye and 1.0 per cent of protein at approximately pH 7.8 was placed in a water bath at 57°C., which is about 10° below the denaturation temperature of the protein. At the end of 3 or 4 days, depending upon temperature, concentration of reagents, and pH, precipitate began to appear. The amount of precipitate increased for several days. After 11 to 14 days the mixture was removed from the bath, placed in a cellophane bag, and dialyzed for 24 hours against 1.0 per cent NaCl solution to remove most of the free dye. Some additional precipitation occurred during this process. The material was then placed in a fresh bag and dialyzed until no more dye was removed against 0.5 N arsenite solution, made by dissolving arsenic acid (*p*-aminophenylarsonic acid, $\text{H}_2\text{NC}_6\text{H}_4\text{AsO}_3\text{H}_2$) in sodium hydroxide solution and bringing to the desired pH, 7.5. This required about 4 days, during which the solution was continually agitated and the hapten solution, the volume of which was five times that of the protein solution, was changed every 24 hours. Most of the precipitate dissolved during this hapten dialysis; the insoluble protein material which remained was discarded and the solution was then dialyzed against 1.0 per cent NaCl solution until free of hapten. This required 3 to 4 days. If all of the dye had not been removed by treatment with hapten precipitation occurred as soon as the hapten concentration was reduced by saline dialysis, and the foregoing process had then to be repeated.

The final solution prepared by this method was clear and contained approximately 40 per cent of the original protein and no dye. The modified protein, which will subsequently be referred to as manufactured antibody, behaved like natural antibody in forming specific precipitates and in the fixation of complement. It was specific in that it reacted with homologous antigens, namely many of the simple phenylarsonic acid azo dyes and azoproteins, but failed to react with similar sulfanilic antigens. Furthermore, preparations of γ -globulin which had been treated in a similar manner but in the absence of dye also failed to show any activity.

Precipitation tests using the trisubstituted resorcinol dye as test antigen gave typical optimum proportion zones, with no precipitate forming in excess antigen or in the presence of homologous hapten such as sodium arsenite. The precipitates which formed with both simple dyes and azoproteins showed the same low solubility as natural antibody precipitates. For example, there

was no appreciable loss of antigen or antibody after the third washing with buffer solution, and diluted antigen-antibody mixtures gave the same amounts of precipitate as undiluted mixtures. However, the reactions differed from those of immune sera in that more critical conditions were necessary for precipitation. For example, the manufactured antibody was found to give no precipitate with antigen at pH values greater than 6.5 or with NaCl concentration over 4 per cent, although formation of soluble complexes of antibody and antigen was shown to have occurred by the failure of the dye antigen to dialyze out of the solution. Another apparent difference was that with increase in pH the optimum proportion zone was found to shift to a higher antibody/antigen ratio. These characteristics are presumably due to the particular method of preparation used.

Precipitin tests were carried out as follows —

To 10 ml portions of manufactured antibody solution containing acetate buffer there were added equal volumes of various dye dilutions which were also buffered with acetate. The mixtures were allowed to stand for 24 hours at room temperature, and the precipitates were then washed three times with buffer solution and analyzed for dye colorimetrically and for total nitrogen by micro Kjeldahl. The antibody nitrogen in the precipitate was then obtained as the difference between total and dye nitrogen. The same methods were employed for the azoprotein antigens.

The results obtained with the trisubstituted resorcinol dye at pH 5.0 and 5.5 are given in Table I. The amount of manufactured antibody was kept constant (4.10 mg) and the amount of dye was varied from 5.280 mg to 0.020 mg. At pH 5.0 the equivalence zone occurred at 1.320 mg of dye and at pH 5.5 the zone shifted to 0.330 mg. However, the equivalence zone for a given pH occurred at a constant total antibody/antigen ratio over a wide range of antibody dilutions. The molecular ratio of antibody to antigen in the precipitates was much smaller than is ordinarily obtained with natural antibody, but several factors make the significance of the values doubtful. Of these the most important is that there is a tendency for this dye to associate in the lower pH range. There is also the possibility that the valence of the antibody formed under these conditions differs from that formed under natural conditions due to the randomness of the structural changes which take place during denaturation. The titer of this particular preparation of manufactured antibody expressed in terms of limiting dilutions was 1:200,000.

It will be noted from Table I that under the best conditions only about 15 per cent of the manufactured antibody was precipitated. However, we might well expect that the methods described above would produce a very heterogeneous mixture of protein structures varying from unchanged molecules and univalent antibodies to antibodies with several combining regions. A more active preparation with about 80 per cent of the protein precipitable could be

TABLE I

Data for Precipitation Reactions of Phenylarsonic Acid Azodye and Manufactured Antibody

Tube No.	Amount of antibody used	Amount of antigen used	Composition of precipitate		
			Amount of dye	Amount of antibody	Molecular ratio antibody/antigen
	mg	mg	mg	mg	
pH 5.0					
1	4.10	5.280	0.106	0.387	0.020
2	"	2.640	0.111	0.591	0.027
3	"	1.320	0.120	0.617	0.026
4	"	0.660	0.099	0.548	0.029
5	"	0.330	0.077	0.465	0.032
6	"	0.165	0.048	0.362	0.039
7	"	0.082	0.027	0.248	0.048
8	"	0.041	0.021	0.158	0.039
9	"	0.020	0.016	0.135	0.045
pH 5.5					
1	"	5.280	0.022	0.070	0.016
2	"	2.640	0.047	0.169	0.018
3	"	1.320	0.053	0.232	0.022
4	"	0.660	0.054	0.259	0.024
5	"	0.330	0.039	0.304	0.040
6	"	0.165	0.032	0.190	0.030
7	"	0.082	0.021	0.156	0.038
8	"	0.041	0.009	0.089	0.050
9	"	0.020	0.009	0.056	0.032

TABLE II

Data for Precipitation Reactions of Phenylarsonic Acid A.-Ovalbumin and Manufactured Antibody

Tube No.	Amount of antibody used	Amount of antigen used	Composition of precipitate		
			Amount of antigen	Amount of antibody	Molecular ratio antibody/antigen
	mg	mg	mg	mg.	
pH 5.5					
1	4.10	6.530	0.318	0.570	0.67
2	"	3.265	0.256	0.420	0.62
3	"	1.632	0.219	0.407	0.71
4	"	0.816	0.212	0.362	0.65
5	"	0.408	0.100	0.320	1.22
6	"	0.204	0.068	0.212	1.18
7	"	0.102	0.043	0.162	1.43
8	"	0.051	0.043	0.137	1.21

obtained by extracting only the dye-protein precipitate which formed during the initial dialysis against saline, but the yields have been so small that very little work has been done with such preparations. It will also be noted that only about 15 per cent of the dye antigen was precipitated under optimum conditions, which agrees closely with results obtained with the same dye and natural antibodies.

The manufactured antibody solution was found to precipitate azoprotein. Reactions with phenylarsonic acid azo-ovalbumin (Table II) were observed to be somewhat different from those with the simple dye antigen in that under the conditions studied so far no optimum zone has been obtained with respect to change in pH or antibody dilutions. The antigen used was prepared by coupling the diazonium salt of arsanilic acid in the usual manner to crystalline ovalbumin. The resulting product analyzed 74 hapten groups per molecule of protein. Within the range studied, the amount of manufactured antibody protein precipitated was of the same order as that found in the simple dye-antibody precipitates. However, the amount of antigen was much less, and the antibody/antigen ratios are similar to those obtained in precipitates with natural antibody. For example, in the region approximating antigen excess the ratio is 0.67 and in the region of antibody excess the ratio became slightly higher than one.

Experiments on the Manufacture of Antibodies to Pneumococcus Polysaccharide Type III

Similar experiments were also carried out with pneumococcus polysaccharide Type III as antigen.

1 gm. of pneumococcus polysaccharide Type III was kindly sent to us by Dr. Walther Goebel of The Rockefeller Institute for Medical Research. This was used with γ -globulin in the same way as the azoresorcinol antigen. 25 ml. of 1 per cent γ -globulin solution was taken, and 250 mg. of the polysaccharide was dissolved in it, a little alkali also being added, since the polysaccharide was in the form of an acid. The final pH was adjusted to 7.5. The mixture was then kept for 2 weeks at 57° in a thermostat. At the end of this time a small amount of precipitate had been formed. The mixture was removed from the bath and the solution separated from the precipitate. It was found that when a small amount of polysaccharide solution was added to a portion of the supernatant solution a precipitate was formed. This was presumably an antigen-antibody precipitate, indicating that in the original solution there was an excess of antibody. This precipitate was found to contain both protein and polysaccharide, the protein was shown to be present by the biuret test and the polysaccharide by dissolving it in dilute alkali and adding calcium hydroxide, which produced a precipitate of the calcium salt of the polysaccha-

ride Both protein and polysaccharide were also shown to be present in the precipitate originally produced during the 2 weeks' heating

The specificity of the supernatant solution was shown by the fact that it did not precipitate with Type I polysaccharide solution The Type VIII polysaccharide also did not give a precipitate, despite the fact that it contains the same groups as the Type III polysaccharide, this may have been due to weakness of the artificial antiserum

Agglutination tests were also carried out with the original supernatant solution. It was found that this solution agglutinated Type III pneumococci but did not agglutinate either Type I or Type II pneumococci.

The attempt was then made to prepare a pure preparation, by separating the polysaccharide from the protein solutions. This was tried in two ways. The first method tried consisted in adding sodium chloride to about 15 per cent concentration, and then adding hydrochloric acid to about pH 4, at which point the polysaccharide precipitated It was removed, and the solution dialyzed against physiological saline and the pH adjusted to 7 The solution obtained in this way was found to have only slight activity It gave a very slight precipitate with solution of Type III polysaccharide only in a very narrow concentration zone This zone was in the region of a tenth of a milligram of polysaccharide per milliliter of undiluted antibody solution. Polysaccharide Type I did not give any reaction with this antibody solution

The other preparation was made by adding 15 per cent salt, and then calcium hydroxide and calcium chloride which precipitates the polysaccharide The pH was about 9 after this treatment. The resulting solution was found to give a small amount of precipitate with polysaccharide Type III in a narrow concentration zone The reaction was somewhat stronger than that given by the solution made by the acid precipitation method

Agglutination tests were carried out with these purified antibody solutions. The solution from the acid precipitation did not give definite agglutination That from the calcium precipitation gave definite, though not strong agglutination with Type III pneumococci The agglutination was not complete enough to cause all the pneumococci to form a single clump but was enough to cause many small clumps to form. The agglutination could be achieved still after two twofold dilutions of the original purified antibody solution, which contained about 3 mg of protein per milliliter The fact that agglutination did not occur after further dilution shows that the agglutinin solution is very weak. Agglutination could not be detected with either Type I or Type II pneumococci

It seems likely, from the low titer of the agglutinin in the purified antibody solution, that the artificial antibody was largely destroyed by the process of purification Probably the most active fraction was carried down with the polysaccharide during its precipitation.

SUMMARY

A protein solution with the properties of a specific antiserum to the triphenylmethane dye methyl blue has been made by treating a solution of bovine γ -globulin and the dye with alkali and then slowly neutralizing the alkali. Some success has been obtained also in the formation of antibodies from other serum proteins and by other denaturation-renaturation procedures.

By heating solutions of γ -globulin and antigen to 57°C for several days antisera homologous to the antigens have been prepared. This method has been used successfully with the azodye 1,3-dihydroxy-2,4,6-tri(*p*-azophenylarsonic acid)benzene and with pneumococcus polysaccharide Type III. The antipneumococcus sera were found to precipitate the polysaccharide of Type III but not those of Types I and VIII and to agglutinate pneumococci of Type III but not those of Types I and II.

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THE ACTION OF EXTREME COLD ON LEUKEMIC CELLS OF MICE*

By CHARLES BREEDIS M.D.

(From the Department of Pathology, Cornell University Medical College New York)

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Viruses (1), bacteria (2, 3), and single celled or small multicellular animals and plants (4, 5) may survive temperatures of liquid air or lower. The resistance of forms that show marked cold hardiness is correlated with ability to survive desiccation (6a). Experiments attempting to determine the resistance of normal and neoplastic tissues to freezing have given variable results. The methods by which freezing and thawing have been accomplished and the possibility that different types of cells may require for their survival different conditions have received little attention. These aspects of the problem in relation to the agent transmitting leukemia of mice form the subject of the present report.

Michaels (7) froze Jensen's mouse carcinoma in liquid air for a half hour and found that it could still be successfully transplanted. Salvin-Moore and his co-workers (8, 9), Gaylord (10), and later investigators have confirmed this observation with other mammalian tumors. Salvin-Moore and Walker (8) believing tumor cells to be killed by such extreme cold, suggested that resistance to freezing is evidence for the existence in tumors of an extracellular transmitting agent. In support of this hypothesis, Lambert (11), Lake (12), and Simonin (13) were unable to obtain growth in tissue culture from embryonic tissues frozen to -6°C . or lower. Similarly, Cramer (14) could obtain no growth from mouse sarcoma tissues frozen three times to between -20°C and -40°C . though these tissues even when frozen eight times to less than -80°C . still produced tumors on inoculation. Lambert (11b) had observed growth, however, from explants of tumors frozen to -16°C . and Mider and Morton (15) have in recent years found evidence of characteristic cell proliferation in subcutaneously implanted normal rat skin that had been frozen to -74°C . Klinké (16a) has demonstrated growth in tissue culture from fragments of sarcomata and carcinomata of mice, as well as from normal chick embryo heart, that had previously been frozen in liquid air. These observations show that resistance of neoplasms to freezing is not evidence that they are transmitted by a virus.

Studies in this laboratory have shown that frozen tissues of leukemic mice retain their ability to transmit leukemia if freezing has been slow, but are innocuous if freezing has been rapid (17). The malignancy of slowly frozen tissues is unaltered after storage for 2 years at -70°C . (18).

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Few other observers have found slow freezing to be less destructive than rapid freezing. Rahm (4), using rotifers, nematodes, and tardigrades in the wet state, and Iljin (19), using leaves of the red cabbage, had earlier obtained similar results. Other investigators find that the rate makes little difference, or that rapid freezing is better than slow. For the preservation of spirochetes, Turner and Brayton (20) consider only the rate of thawing to be important, and this must be rapid.

Several recent investigations show both rapid freezing and thawing to be the best method for the preservation of certain types of cells. Coggeshall (21) found this to be the case for malarial parasites. Similar results for the preservation of motility of human spermatozoa were obtained by Shettles (22), Jahnel (23), and Hoagland and Pincus (24), and for that of frog spermatozoa by Luyet and Hodapp (25). The irritability of frog muscle fibers and the motility of the vinegar eel, a nematode, could be preserved only in this way by Luyet and his coworkers (6*b*, 26). The rapid freezing method of Luyet differs essentially from that employed by other workers and will be discussed later.

Mider and Morton (15) found the Walker rat sarcoma, Sarcoma 387, and Sarcoma 180 to be equally transmissible whether freezing to -74°C had been slow or rapid, but on repeated freezing slowly frozen tumors gave no takes on inoculation, whereas rapidly frozen tumors could be refrozen five to seven times before they were inactivated. Klinke (16*a*) emphasizes the necessity of rapid freezing and thawing for preserving normal tissues and carcinomata or sarcomata of mice, rats or rabbits.

There is considerable evidence in favor of the view that the agent transmitting leukemia of mice is the live leukemic cell itself (27, 28*a*). If both leukemia and cancer of mammals depend for their transmission on living cells, it is remarkable that these cells should react so differently to a physical agent and that leukemic cells should behave like those of distant species. Consequently it seemed desirable to determine more precisely the freezing conditions required for the inactivation of the leukemia-transmitting agent, or leukemic cell.

Of the eight experiments to be reported, the first four were done to test the effect of very slow and very rapid freezing on the transmissibility of three different strains of leukemia. In the last four, one of these strains was tested under various cooling conditions.

Methods and Materials

Three strains of leukemia were used. Strain Rfb 385 arose from a mouse having monocytic leukemia, induced by intrasplenic injection of benzpyrene (28*b*). Strains Akf 5 and fAlh 1032 are from mice with spontaneous lymphocytic leukemia. All three have been transmitted through many generations, by both intravenous and subcutaneous inoculation.

Cell suspensions to be frozen were prepared by mincing subcutaneous tumor, spleen, and lymph nodes of a leukemic mouse with scissors in Tyrode's solution that contained 10 per cent of rabbit serum or of amniotic fluid. The suspension was cleared of gross particles by sucking it through a small piece of cotton and into a

syringe through a 27 gauge needle. The concentration of leukemic cells was then determined in a counting chamber

In the first four experiments thin walled glass tubes (Fig. 1, A) were used for freezing. Suspensions to be frozen slowly were transferred from the syringe to the bottoms of the tubes through the 27 gauge needle. The tubes were then placed in an alcohol bath at 0°C. The temperature of the bath was lowered at a rate of approximately 0.5° per minute by dropping into it small pieces of solid CO₂. At -60°C the rate was increased to 1° per minute, and at -70° the tubes were immersed in liquid nitrogen at -196°

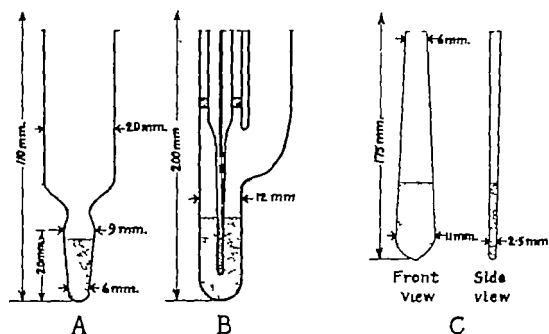


FIG 1 Types of glass tubes used for freezing leukemic cell suspensions. Wall thicknesses are A, 0.5 mm. B, 1 mm., C, 0.2 mm. The amounts of suspension are 0.8 cc., 1 cc., and 0.4 cc. respectively

Rapid freezing was done by allowing the suspension to fall drop by drop from the end of the same 27 gauge needle onto the inner wall of the same type of tube immersed in liquid nitrogen. The tube was held at an angle, and its position was frequently changed so that each drop fell on a fresh area of wall in the wide upper portion of the tube, where it froze within a second or two

All tubes were kept at -196° for approximately an hour and were thawed as needed for injection. Slow thawing was done by placing the tube in a small beaker containing approximately 20 cc. of alcohol at -40° in the ice box. Thawing was complete in 20 to 30 minutes. For rapid thawing the tube was transferred to alcohol at -40° for 5 minutes and was then shaken in water at 37°. Thawing was complete in 8 to 12 seconds.

Injection of Mice—A tube containing the original suspension was kept in ice water until the other tubes had been frozen. Then a series of dilutions was prepared for injection, using Tyrode's solution containing 10 per cent of rabbit serum or of amniotic fluid as the diluent. The frozen and thawed suspensions were injected

without dilution All injections were intravenous, 0.1 cc. being given into the tail vein of each mouse

The procedures in Experiments 5 to 8 will be described separately

The Effect of Rapid or Slow Freezing or Thawing
(Experiments 1 to 4)

The procedure in all four of the experiments was the same, though each was done on a separate day and at different times of the year Three strains of leukemia were tested

The results of inoculations with suspensions frozen and thawed as described are shown in Tables I to IV, and will be considered together

TABLE I

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain Rfb 385, Frozen to -196°C (Experiment 1)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	days
Not frozen	3,500,000	5	4	14-17	15.5
Not frozen	35,000	5	3	20-34	25.7
Not frozen	350	6	6	28-40	33.0
Not frozen	3.5	6	0	—	—
Slow frozen, slow thawed	3,500,000	6	0	—	—
Slow frozen, rapid thawed	3,500,000	4	2	29-56	43
Rapid frozen, slow thawed	3,500,000	6	0	—	—
Rapid frozen, rapid thawed	3,500,000	6	0	—	—

All of the 8 rapidly frozen suspensions, injected into a total of 48 mice, were inactive, whereas 5 of the 7 slowly frozen suspensions were still capable of transmitting leukemia to 20 of the 38 mice injected

When methods of thawing are compared, all of the 4 slowly frozen and rapidly thawed suspensions are active, in 15 of 22 mice, as compared to only one of the 3 slowly frozen and slowly thawed suspensions, in 5 of 16 mice Unfortunately, one tube of the latter material (Table IV) cracked during freezing and was not injected

The average length of life after inoculation gives a fair indication of the concentration of the transmitting agent, as seen from the results of injecting diluted suspensions (Tables I to IV), and may be used to estimate the destructive effect of the freezing process On this basis the frozen and thawed suspensions from the three stains of leukemia tested correspond in potency to the following dilutions of the fresh suspensions strain Rfb 385 (Tables I and

TABLE II

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain Rfb 385 Frozen to -196°C (Experiment 2)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	days
Not frozen	2 200 000	5	5	18-22	19.6
Not frozen	22 000	6	4	33-37	34.7
Not frozen	220	6	0	—	—
Slow frozen slow thawed	2 200 000	5	0	—	—
Slow frozen rapid thawed	2 200 000	6	1	37	37
Rapid frozen slow thawed	2 200 000	6	0	—	—
Rapid frozen rapid thawed	2,200 000	6	0	—	—

TABLE III

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain Akf 5, Frozen to -196°C (Experiment 3)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	days
Not frozen	1 800 000	5	4	10-14	11.0
Not frozen	18 000	5	5	14-14	14.0
Not frozen	180	5	5	15-21	17.4
Not frozen	18	4	2	15-33	24
Slow frozen slow thawed	1 800 000	5	5	14-15	14.8
Slow frozen, rapid thawed	1 800 000	6	6	14-15	14.5
Rapid frozen slow thawed	1 800 000	6	0	—	—
Rapid frozen rapid thawed	1 800 000	6	0	—	—

TABLE IV

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain fAkh 1032 Frozen to -196°C (Experiment 4)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	days
Not frozen	6 000 000	6	6	6-9	7.0
Not frozen	60 000	6	5	8-10	9.4
Not frozen	600	6	6	12-19	13.1
Not frozen	6	6	6	14-44	25.7
Slow frozen rapid thawed	6 000 000	6	6	11-13	11.3
Rapid frozen, slow thawed	6 000 000	6	0	—	—
Rapid frozen rapid thawed	6 000 000	6	0	—	—

II), slow frozen rapid thawed, between 1 10,000 and 1 1,000,000 in the first experiment and between 1 100 and 1 1000 in the second, strain Akf (Table III), slow frozen and slow or rapid thawed, about 1 100, strain fAk 1032 (Table IV), slow frozen rapid thawed, between 1 100 and 1 1000

Though these survival values are small, they still correspond to from 100 to 1000 times the smallest infectious dose for strains Akf 5 and fAk 1032. Strain Rfb 385 is less resistant to freezing, and barely enough agent is preserved to transmit the disease

Temperature Changes during Slow Freezing

(Experiment 5)

It is well known that slowly cooled aqueous solutions may not freeze at their true freezing points, and can often be supercooled many degrees. Supercooled leukemic cell suspensions are fairly stable at temperatures above -9°C . When inoculated with ice they rapidly congeal. In the previous four experiments freezing of the slowly cooled suspensions occurred spontaneously between -9° and -13° .

Since the formation of ice results of the liberation of a large amount of heat, there is a marked rise in temperature when a supercooled suspension freezes. Consequently, lowering of the bath temperature at a constant rate will not result in a similar rate of lowering of the temperature of the suspension while freezing is going on.

The present experiment was undertaken to determine whether supercooling until freezing takes place spontaneously is more or less deleterious than freezing at the freezing point following inoculation with ice.

Howard (29) has found that the freezing points of normal and neoplastic rat tissues are almost identical, and are of the order of -0.6°C .

Procedure—The cell suspension was prepared as in the previous four experiments, in Tyrode's solution containing 10 per cent of rabbit serum. Strain fAk 1032 was used.

Four special Pyrex glass tubes (Fig 1, B), each containing 1 cc of suspension, were immersed in a mechanically stirred alcohol bath in a thermos bottle. In the center of each tube, immersed in the suspension, was placed a calibrated copper-constantan thermocouple, the other junction of which was kept in ice water. The bath temperature was followed with a thermocouple and a pentane thermometer.

The bath was cooled or warmed at a slow, uniform rate by allowing heat to flow into it or out through a heavy U-shaped copper bar, one prong of which dipped into the bath and the other into another thermos bottle filled with either warm water or solid CO_2 in alcohol.

The course of the temperatures of the four suspensions as the bath was slowly cooled is shown in Fig 2. They followed the temperature of the bath with a constant lag of approximately 0.5°C , except in two regions. At -1°C the suspensions in

tubes 1 and 2 were inoculated with a small amount of Tyrode's solution that had been frozen on the end of a fine capillary and introduced through the side arm of the tube.

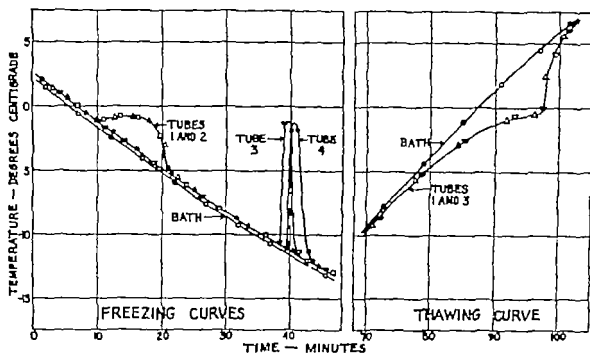


FIG 2 Temperature changes in leukemic cell suspensions during slow freezing and thawing. Results of injection are shown in Table V

TABLE V

*Effect of the Method of Slow Freezing to -21°C on Cells of Strain f Akh 1032
(Experiment 5 Cf Fig 2)*

Material injected	No of mice		Length of life after injection	
	Injected	Died with leukemia	Extremes	Average
			days	days
Tube 1 Frozen at -1°C near freezing point, slow thawed	6	6	10-12	11.0
Tube 2 Frozen at -1°C . near freezing point, rapid thawed	6	6	10-11	10.5
Tube 3 Supercooled, freezing at -10.7°C , slow thawed	6	6	10-12	11.0
Tube 4 Supercooled, freezing at -11.2°C . rapid thawed	6	6	9-10	9.8
Not frozen	6	6	7-11	8.2

The temperature of both suspensions lagged behind that of the bath and the other two tubes until freezing was nearly complete, at about -5° . The suspensions in tubes 3 and 4 began to freeze spontaneously, at -10.7° and -11.2° respectively, as can be seen from the sudden rise in their temperature from these points almost to

their freezing point and its subsequent rapid return to the temperature of the other two suspensions, which had followed the bath temperature at a constant rate.

The temperature of the bath was allowed to drop slowly to -21° . Tubes 2 and 4 were then thawed rapidly by shaking them in water at -37° . Thawing was complete in approximately 30 seconds. Tubes 1 and 3 were kept in the bath as its temperature was slowly raised. Both tubes followed the same thawing curve, shown in Fig. 2. It is evident that thawing commenced at least 5° below zero and reached its most rapid rate between -1° and 0° .

The thawed suspensions were injected intravenously into mice in amounts of 0.1 cc., each mouse receiving an estimated 4,000,000 cells.

Results of inoculations with each of the four frozen suspensions and a fresh suspension are shown in Table V. All of 30 mice inoculated died of leukemia in from 7 to 12 days. Since the average length of life does not differ significantly among the four groups receiving frozen material, it is probable that the destructive effect of each of the methods of freezing and thawing is about the same. On comparison with the average length of life of mice receiving fresh material, and with the results of the previous experiment using this strain of leukemia (Table IV), it is estimated that again only a few per cent of the transmitting agent was preserved.

The Effect of Rapid Cooling through Various Temperature Ranges

(Experiment 6)

In this experiment it was attempted to find whether the lethal action of rapid cooling is limited to a definite temperature range, and to estimate the rapidity of cooling necessary to render leukemic cell suspensions innocuous.

Procedure—A leukemic cell suspension was prepared as in the previous experiment, using strain fAkh 1032. Freezing was done in nine flat glass tubes having very thin walls (Fig. 1, C), so that the suspension would rapidly take on the temperature of the surrounding bath. Each tube contained 0.4 cc. of suspension.

Three baths in thermos bottles were set up, ice water at 0°C , liquid nitrogen at -196°C , and an ether bath. The last was cooled at a slow constant rate of 1°C per minute by dropping into it small pieces of solid CO_2 .

The method of cooling the suspensions is shown diagrammatically in Fig. 3, where the course of temperature of each of the nine tubes is represented by a numbered heavy line. The horizontal portion of a line represents the suspension in one of the constant temperature baths, the oblique portion slow cooling at a rate of 1° per minute, and the vertical portion rapid cooling following removal from one bath and immersion in another at a lower temperature.

Tube 1 was immersed in liquid nitrogen from a temperature of $+37^{\circ}\text{C}$. Tubes 2, 3, 4, 5, and 6 were placed in the ether bath at 0° and were immersed in liquid nitrogen when their temperature had reached 0° , -8° , -15° , -40° , and -70° respectively. Tubes 7, 8, and 9 were taken from the ice water bath and immersed in the ether bath when its temperature was -15° , -40° , and -70° respectively. A few minutes after the ether bath had reached -70° these tubes were immersed in liquid nitrogen.

All of the tubes were kept in liquid nitrogen for an hour, and were then thawed rapidly for injection by immersing them in water at room temperature. Injections were intravenous, each mouse receiving 0.1 cc. of suspension containing 3,000,000 cells.

The rapid freezing method of vitrification described by Luyet (see discussion) was also attempted with this cell suspension. Approximately 15 large glass coverlips of No. 1 thickness and 24 by 40 mm. in size were immersed in the suspension, taken out one by one, and rapidly plunged into liquid nitrogen. Material so frozen onto the coverlips had a glassy transparency when held toward the light. On warming a few seconds in the air it suddenly became white and opaque (devitrification). A few seconds later, just before thawing set in it became clear again.

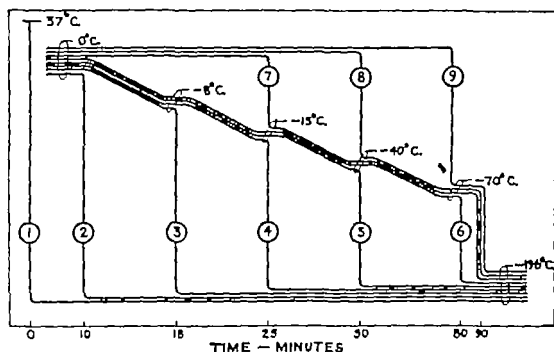


FIG. 3 Diagram showing temperature changes to which 9 samples of leukemic cell suspension were subjected. Results of injection are shown in Table VI.

The material was rapidly thawed by dropping the coverlips, one by one into Tyrode's solution containing 10 per cent of rabbit serum at room temperature. The resulting suspension was injected intravenously in amounts of 0.3 cc. into 5 mice, each receiving an estimated 500,000 cells.

Results of the inoculations are shown in Table VI. Tubes 1, 2 and 3, immersed in liquid nitrogen from temperatures of $+37^{\circ}$, 0° , and -8° respectively failed to produce leukemia in any of the 14 mice injected. Tubes 4, 5 and 6, slowly cooled to temperatures of -15° , -40° , and -70° respectively and then immersed in liquid nitrogen, produced leukemia in all of the 15 mice injected. Tube 7, rapidly cooled to -15° , then slowly to -70° , and finally rapidly to -196° , produced leukemia in all of 5 mice injected, whereas tubes 8 and 9, where the initial rapid drop was to -40° and -70° respectively,

were innocuous in the total of 9 mice injected. The vitrified material also failed to produce leukemia.

From the results of injecting tubes 1 to 7 it is evident that suspensions which have been brought to a temperature of -15° or less by relatively slow cooling can no longer be inactivated by subsequent rapid cooling. Also, the results of injecting tubes 7, 8, and 9 indicate that the initial drop from 0° to -15° is not rapid enough to inactivate the suspension, whereas the more rapid rate of cooling in dropping to -40° or lower is sufficient. These results suggest

TABLE VI

Effect of Rapid Cooling through Various Temperature Ranges on Cells of Strain fAkh 1032, Frozen to -196°C (Experiment 6 Cf Fig 3)

Material injected, method of cooling	No. of mice		Length of life after injection	
	Injected	Died with leukemia	Extremes	Average
			days	days
Tube 1 Rapid, $+37^{\circ}$ to -196°	5	0	—	—
Tube 2 Rapid, 0° to -196°	4	0	—	—
Tube 3 Slow, 0° to -8° , rapid, -8° to -196°	5	0	—	—
Tube 4 Slow, 0° to -15° , rapid, -15° to -196°	5	5	10-11	10.6
Tube 5 Slow, 0° to -40° , rapid, -40° to -196°	5	5	10-19	12.8
Tube 6 Slow, 0° to -70° , rapid, -70° to -196°	5	5	10-12	11.0
Tube 7 Rapid, 0° to -15° , then slow to -70° and rapid to -196°	5	5	12-13	12.2
Tube 8 Rapid, 0° to -40° , then slow to -70° and rapid to -196°	5	0	—	—
Tube 9 Rapid, 0° to -70° , rapid -70° to 196°	4	0	—	—
Vitrified, 0° to -196°	5	0	—	—
Not frozen	5	5	7-10	8.0

that in rapid cooling from 0° to -196° , as in the case of tube 2, the lethal change is complete by the time -40° is reached and that most of it takes place in the range 0° to -15°C .

The initial temperature changes in the rapid drop from 0° to -196° and from -8° to -196° were determined later (cf Fig 5).

Effect of Rapid Cooling on Frozen and Supercooled Suspensions at the Same Temperature

(Experiment 7)

The question arises as to whether complete inactivation occurs because of rapid passage through a definite temperature range or is due to rapid transition of the material from the liquid to the solid state, at whatever temperature this

may occur. In all of the previous experiments, slowly cooled suspensions were still liquid (supercooled) at $-9^{\circ}\text{C}.$, and froze spontaneously between this temperature and -15° . Since it is possible to obtain liquid or frozen suspensions at the same temperature, the latter by simply inoculating the liquid suspension with ice, and then subject both to rapid cooling one may decide between these two possible causes of inactivation.

Procedure—Strain fAlh 1032 was again used, and a cell suspension was prepared as in the previous experiment.

Eight of the same type of flat tubes each containing 0.4 cc. of suspension, were brought through the various temperature curves shown diagrammatically in Fig. 4.

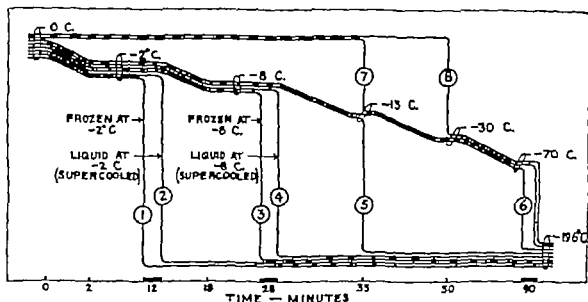


FIG. 4 Diagram showing temperature changes to which 8 samples of leukemic cell suspension were subjected. Results of injection are shown in Table VII.

Three baths were set up, ice water at 0° , liquid nitrogen at -196° , and an ether bath. The last was slowly cooled from 0° to -70° at a constant rate of 1° per minute, except at -2° and -8° , where it was held constant for 10 minutes at each temperature.

Tubes 1 to 6 were placed in the ether bath at 0° and cooling of the bath was started. At -2° the suspension in tube 1 was inoculated with a small amount of Tyrode's solution frozen on the end of a fine capillary. The suspension froze rapidly and appeared solid within 30 seconds. The bath was maintained at -2° for 10 minutes. At the end of this time tube 1, its suspension frozen solid, and tube 2 its suspension still liquid were immersed in liquid nitrogen. Tubes 5 and 6 froze spontaneously at -14° and -12° respectively. Tube 5 was immersed in liquid nitrogen when the temperature of the bath was -15° , tube 6 when it was -70° . Tubes 7 and 8 were taken from ice water and immersed in the ether bath when its temperature reached -15° and -30° respectively. When it reached -70° both were immersed in liquid nitrogen.

Solid particles of spleen and lymph node were frozen in an additional two of the flat tubes, not shown in Fig. 4. This was done in order to test whether inactivation

by rapid freezing is peculiar to cell suspensions alone. One tube was frozen rapidly by immersion in liquid nitrogen from a temperature of 0° . The other was subjected to the same slow freezing conditions as tube 6.

All of the tubes were kept in liquid nitrogen for approximately an hour and were thawed rapidly by immersion in water at room temperature. The suspensions in tubes 1 to 8 were diluted to 0.8 cc. and were injected intravenously in amounts of 0.15 cc., each mouse receiving a total of 2,000,000 cells. Cell suspensions were prepared from the thawed particles of spleen and lymph node. These were injected intravenously in amounts of 0.1 cc., each mouse receiving an estimated 500,000 cells.

TABLE VII

Effect of Rapid Cooling on Frozen and Supercooled Suspensions at the Same Temperature, Strain fAkh 1032, Frozen to -196°C (Experiment 7 Cf Fig 4)

Material injected, method of freezing	No. of mice		Length of life after injection	
	Injected	Died with leukemia	Extremes	Average
			days	days
Tube 1 Frozen at -2° , then immersed in liquid nitrogen	5	1	16	16
Tube 2 Liquid at -2° , then immersed in liquid nitrogen	5	0	—	—
Tube 3 Frozen at -8° , then immersed in liquid nitrogen	5	5	12-13	12.4
Tube 4 Liquid at -8° , then immersed in liquid nitrogen	5	0	—	—
Tube 5 Slow, 0° to -15° , rapid, -15° to -196°	5	5	11-12	11.2
Tube 6 Slow, 0° to -70° , rapid, -70° to -196°	4	4	11-15	12.5
Tube 7 Rapid, 0° to 15° , slow, -15° to -70° , rapid, -70° to -196°	5	5	11-12	11.2
Tube 8 Rapid, 0° to -30° , slow, -30° to -70° , rapid, -70° to -196°	5	1	15	15
Solid particles, rapid 0° to -196°	5	0	—	—
Solid particles, slow, 0° to -70° , rapid, -70° to -196°	6	6	12-13	12.2
Not frozen	5	5	8-10	8.6

The results of injection with these frozen and thawed materials are shown in Table VII.

As was expected from the previous experiment, the suspensions in tubes 5 and 6, which had already been frozen to -15° and -70° respectively before immersion in liquid nitrogen, produced leukemia in all of the 10 mice injected.

The suspensions in tubes 2 and 4, supercooled to -2° and -8° respectively and then immersed in liquid nitrogen, were innocuous for all of the 10 mice injected. On the other hand, the suspensions in tubes 1 and 3, which had been inoculated with ice and allowed to freeze at these respective temperatures

before immersion in liquid nitrogen, still retained their ability to transmit leukemia. The former produced the disease, after a delayed incubation period, in one of 5 mice, the latter in all of 5 mice.

The suspension in tube 7, rapidly cooled from 0° to -15° , then slowly to -70° and finally rapidly to -196° , produced leukemia in all of 5 mice injected, whereas that in tube 8, where the initial rapid drop was from 0° to -30° , produced leukemia in only one of 5 mice after a delayed incubation period. It will be recalled that in Experiment 6 the suspension subjected to an initial rapid temperature drop from 0° to -40° was innocuous.

The results of Experiments 6 and 7 indicate that to be protected from the destructive effect of rapid cooling suspensions must previously be frozen whether spontaneously or by inoculation with ice. The initial temperature of the frozen suspension is also important, as seen from the results of injection of the three suspensions in Experiment 7 that were frozen in baths at -2° , -8° , and -15°C and then immersed in liquid nitrogen. The first is almost innocuous; only one of 5 injected mice died of leukemia, after 16 days. Mice injected with the second and third suspensions all died, after an average length of life of 12.4 and 11.2 days respectively. It appears that the agent is better protected from rapid cooling by previous freezing at the lower temperatures. The thawing curve shown in Fig. 2 (Experiment 5) suggests that frozen suspensions still contain a considerable amount of unfrozen water at -2° .

Rapid and slow freezing had the same effect on solid particles of spleen and lymph node as on the suspensions. The 5 mice injected with rapidly frozen material failed to develop leukemia, whereas the 6 injected with slowly frozen material all died of the disease.

The initial temperature change in the rapid drop from 0° to -196° and from -8° to -196° were determined later (*cf* Fig. 5). It is of interest that in the second case the temperature at first rose to the freezing point despite the rapid cooling action of the bath.

Temperature Changes during Rapid Freezing The Rate of Cooling Necessary for Inactivation

(Experiment 8)

Since frozen suspensions at -2° , -8° , and -15° resist inactivation by subsequent rapid cooling to -196° while liquid suspensions at 0° , -2° , and -8° do not, it is highly probable that the inactivation due to rapid cooling takes place during rapid transition from the liquid to the solid state, somewhere in the range 0° to -15° . Consequently, measurements of the rate of cooling necessary for inactivation would seem to have their greatest significance in this range.

The previous two experiments show that for the type of tube used (Fig. 1, C) a rate of cooling sufficiently great to render the suspensions innocuous

can be attained by transferring the tube from one bath at 0° to another (ether) at -40° . Similar cooling from 0° to -15° results in uniformly active material, even though the temperature is subsequently brought to -196° . Measurements were therefore confined to suspensions originally at 0°C transferred to baths at -15° , -25° , -35° , and -45°C .

Procedure—One of the flat tubes (Fig 1, C) was fitted with a small copper-constantan thermocouple. The thermocouple was held in the center of the suspension by means of cork rings shaped to fit the upper part of the tube, the arrangement being similar to that shown in Fig 1, B. The other junction was kept in ice water.

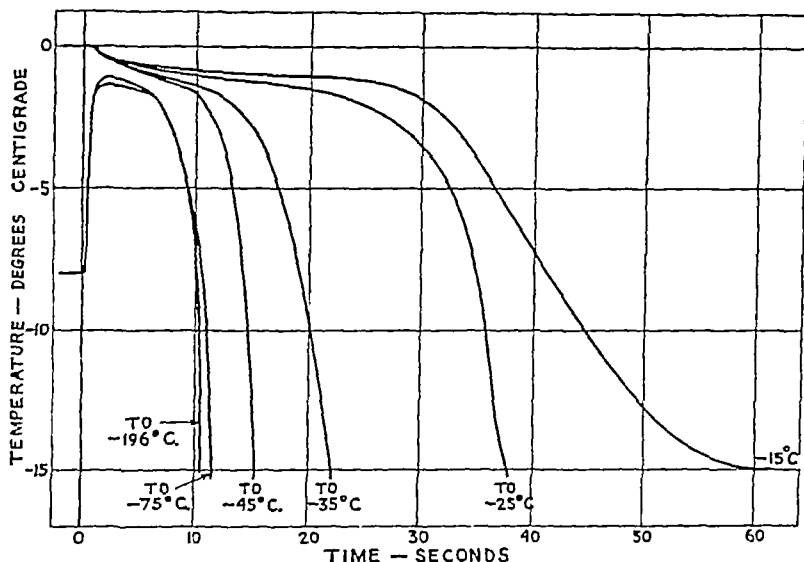


FIG 5 Graph showing temperature changes in the range 0°C to -15°C undergone by leukemic cell suspensions suddenly subjected to freezing baths at various temperatures. Results of injection with all but the two samples initially supercooled to -8°C are shown in Table VIII.

Temperature changes during freezing were recorded by an electrocardiograph machine of the string galvanometer type, manufactured by the Cambridge Instrument Company. The thermocouple was connected directly to the leads of the machine. Calibration was done through the range, 0° to -20° . The recordings were made with the advice and assistance of Mr. R. K. Waller of the Flushing Hospital Laboratory.

A cell suspension was prepared as in the previous experiment, strain fAlh 1032 again being used. The same tube and thermocouple were used to successively freeze four different samples of the suspension. Freezing was done by keeping the tube in ether at 0° for 5 minutes and then rapidly transferring it to ether at -15° , -25° , -35° , or -45° , where it was kept for 15 minutes. It was then thawed by immersion

in water at 25° and injected intravenously in amounts of 0.1 cc., each mouse receiving an estimated 2 000 000 cells. A fresh suspension was kept in ice water until the frozen materials had been injected. It was then injected in a series of dilutions.

A time-temperature recording was made during the freezing of each of the four samples. These curves are shown in Fig. 5. The figure also shows two curves for suspensions that were first supercooled to -8° and then immersed in freezing baths, one ether at -75° and the other liquid nitrogen at -196°. These suspensions were not injected.

The results of injection with frozen samples are shown in Table VIII. Material frozen to -15° produced leukemia in all of 9 mice injected, the mice dying after an average of 8.4 days. Freezing to -25°, -35°, and -45° increased the average length of life to 10.4, 14.0, and 15.3 days respectively,

TABLE VIII

Effect of Freezing on Suspensions Subjected to Cooling Baths at Various Temperatures. Strain fAkh 103² (Experiment 8 Cf Fig 5)

Material injected, method of freezing	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				<i>days</i>	<i>days</i>
0° to -15	3 000 000	9	9	8-9	8.4
0° to -15°	30 000	5	5	9-11	10.2
0° to -25	3 000 000	5	5	10-11	10.4
0° to -35	3 000 000	5	5	13-18	14.0
0° to -45°	3 000 000	10	3	14-16	15.3
Not frozen	3 000 000	6	6	6-6	6.0
Not frozen	30 000	4	4	9-9	9.0
Not frozen	300	5	5	11-12	11.4

and only 3 of the 10 mice injected in the last group died of leukemia. These figures correspond to the following dilutions of fresh material: 1:1 to 1:100 (-15°), 1:100 to 1:10,000 (-25°), less than 1:10,000 (-35°), considerably less than the former (-45°). The survival of material in the last tube was unexpected. Experiments 6 and 7 suggest, however, that if these materials had been subsequently cooled to -196° their potency would have been further reduced and the last would have become innocuous.

The freezing curves for these four materials and for two supercooled suspensions that were not injected are shown in Fig. 5. It is evident from the curves that the interior of the suspension did not become supercooled in any of the tubes. Freezing began at the true freezing point, and the temperature remained high until freezing was nearly complete. Then it dropped rapidly toward the bath temperature. The suspensions already supercooled to -8°

behaved similarly. Their temperatures rose in less than a second to between -1° and -2° , where they remained for some 8 seconds and then began to drop rapidly. It is of interest that the curves for the two supercooled materials are almost identical, though one was immersed in ether at -75° and the other in liquid nitrogen at -196° . Gas from the boiling nitrogen around the tube acts as an insulating layer so that liquid nitrogen is prevented from coming in contact with the tube. This phenomenon does not occur in the case of ether, and the full temperature difference between it and the interior of the tube is utilized.

The times taken for the suspensions to reach a temperature of -15° depended upon the bath temperature to which each was subjected. The suspension taken from the bath at 0° and immersed in a bath at -15° required 60 seconds to reach a temperature of -15° . When the temperature of the second bath was -25° , -35° , -45° , or -196° , the time required for the suspension to reach -15° was 38, 22, 16, and 13 seconds respectively. Similarly, suspensions originally at -8° reached -15° in 12 or 11 seconds when immersed in baths at -75° and -196° respectively. The suspension cooled from 0° to -196° is not represented in the graph, but it followed the same type of curve as the others starting at 0° .

The results of injections in this experiment indicate that the lethal effect of freezing is almost complete by the time -45° has been reached, and that most of it occurs in the narrower range, 0° to -15° . Experiments 6 and 7, combined with the temperature measurements done in this experiment, show that if the temperature of the central parts of a suspension passed through the latter range in 13 seconds or less, the suspension becomes innocuous, whereas if 60 or more seconds are required, approximately 1 per cent of the activity is preserved, provided that the final temperature reached is -196° in both cases.

DISCUSSION

The mechanism of death by freezing has been a subject of considerable study and speculation. There are two prevailing theories, both involving the formation of ice. (1) Ice is first formed extracellularly, causing death by mechanical compression and injury of the cells (30), (2) ice is formed intra- or extracellularly. Its formation involves the withdrawal of water from protoplasm and leads to irreversible changes, due to simple dehydration, change in pH, concentration of toxic substances or other, unknown factors (31). Iljin (19) believes that death may result from too rapid thawing, injury being caused by rapid invasion of protoplasm by water from the melting ice. Luyet and Gehenno (6c) have reviewed several theories, and conclude from their own and previous work that the formation of ice crystals is the primary cause of cell death. Ice crystal formation can be prevented or reduced in several ways. Moran (32a) has found that if gelatin gel is dehydrated until its water content

is 35 per cent or less, ice crystals will no longer form in it. This observation may aid in explaining the extreme resistance to cold of forms that survive desiccation. Cold resistance in plants is correlated with such factors as osmotic value of cell content, percentage of reducing sugars, amount of coagulable nitrogenous constituents, and quantity of dry matter (33).

The success of rapid freezing has been attributed to the fact that smaller ice crystals are formed in this way. Luyet and Gehenio (6*b*) believe that with sufficiently rapid cooling ice crystal formation can be abolished altogether. Their observations indicate that ice crystals form in protoplasm only within a definite temperature range, lying between the freezing point and -40°C . Minute pieces of tissues cooled through this range with extreme rapidity congeal to form amorphous, glass-like solids. The authors term this process "vitrification." Thawing must also be extremely rapid. If vitrified material is kept for even a short time at temperatures between the freezing point and -40°C ice crystals form in it (devitrification). It is of interest in this connection that Moran (32*b*), by means of conductivity measurements, has determined the eutectic temperature of frog muscle to be -37.5°C .

Few investigators have attempted to estimate rapidity of freezing with any degree of exactness, so that much of the variability in results may be due to differences in technique alone. There is no doubt, however, that the vitrification method is far more rapid than any yet employed in investigations on tumor tissue.

The criteria by which survival has been established in materials frozen by vitrification are open to criticism. Epidermal cells of the onion were assumed to have survived freezing on the basis of their staining reaction with neutral red and their osmotic properties (34), frog spermatozoa (25), human spermatozoa (24), and the vinegar eel, a nematode (26), on the basis of motility, frog muscle fibers (6*b*) on the basis of irritability and osmotic properties. It has not been shown in these studies that the cells are able to multiply, or, in the case of spermatozoa, to fertilize ova. Experiments in which growth is observed after freezing or an agent of transmissible disease multiplies *in vivo* after freezing *in vitro* demonstrate survival more conclusively. Turner and Brayton (20) found no close correlation between the motility and infectiousness of frozen and thawed spirochetes.

Results that show slow freezing to be less destructive than rapid freezing are difficult to explain. A number of observations indicate that profound damage occurs when frozen materials are maintained for a protracted time at temperatures close to the freezing point. Moran (32*c*) has found a critical temperature of -2°C at which irreversible changes begin to occur in frog muscle. Freezing to equilibrium at -2° removed 78 per cent of the water as ice. Below this temperature there is loss of irritability and osmotic properties on thawing, and shortening up to 80 per cent occurs. The last change is

prevented by slow thawing. At the critical temperature there is also a sharp maximum of lactic acid formation (35), and maximum precipitation of denatured protein (36).

Haines (3) found that storage of bacteria, and Turner (37) that storage of spirochetes at temperatures between -20°C and 0°C results in their inactivation, more rapid at the higher temperatures. Spirochetes stored at -78°C had not lost virulence after 3 years (38). Frozen tumors stored at -20°C lost their transmissibility in 3 weeks (Klinke, 16*b*). In the methods of slow freezing ordinarily employed the time of passage from 0°C to -20°C is relatively short, so that the types of changes occurring in storage are probably negligible.

Ice formation during slow freezing differs from that during rapid freezing. Chambers and Hale (39) with frog muscle and other investigators (31) with plant tissues observed microscopically that during slow freezing ice grows along the cellular interspace, the cell membrane acting as a barrier. During rapid freezing ice crystals form inside as well as outside of cells. Similar observations were made by Moran (32*a*) on discs of gelatin gel. When gels having a concentration higher than 12 per cent were frozen slowly, ice formed only on the outside of the discs, which contracted as water diffused to the surface and froze. A final equilibrium gel concentration in the discs of 65 per cent was reached at -19°C . Ice crystals did not then form even after immersion in liquid air. During rapid freezing, on the other hand, numerous foci of ice crystal formation were observed throughout the discs.

These observations may to some extent explain the superiority of slow freezing found in the present experiments. Initial freezing of water in extracellular fluids would result in the concentration of osmotically active material outside of the cell. Water would diffuse out of the cell to restore osmotic equilibrium, leaving the protoplasm partially dehydrated. That such protoplasm may be a less favorable site for ice crystal formation has already been indicated. Since the transfer of water would require an appreciable time, it could not occur during very rapid freezing. Though some observers account for cell death, rather than survival, on this basis, it has been found that previous treatment with hypertonic solutions increases the resistance of some forms to freezing (6*c*, 25, 26, 34). This explanation does not account for the difference in reaction of carcinoma and sarcoma cells on the one hand and leukemic cells on the other. The fact that the former are bound down in a tissue matrix while the latter, even in tissue, are surrounded by fluid may be of significance.

The chief interest in the curves showing different rates of cooling is that they are similar in form and differ only in steepness. The sharp endpoint at which the cooling rate causes complete inactivation is remarkable. Approximately 1 per cent of activity was preserved whether cooling through the range 0° to -15°C required 30 minutes or 1 minute, but when this range was passed

through in 12 seconds or less, the material became innocuous, its activity being reduced to less than 0.0001 per cent.

SUMMARY

Suspensions of leukemic cells of mice from three different strains of leukemia were subjected to rapid or slow freezing and rapid or slow thawing.

Suspensions rapidly frozen to -196°C were in all cases innocuous, whereas those frozen slowly were capable of transmitting leukemia. The infectivity of slowly frozen material varied from an estimated 0.0001 per cent to 1 per cent of that of fresh material, and this figure probably represents the percentage of surviving leukemic cells.

Particles of spleen and lymph node reacted to slow and rapid freezing in the same manner as suspensions prepared from them.

For one of the strains rapid thawing was less injurious than slow thawing, for the other two the rate of thawing seemed to be immaterial.

Infectivity was equally well preserved after freezing to -21°C whether freezing occurred spontaneously after supercooling or was initiated near the freezing point by inoculation with ice, or whether thawing was slow or rapid.

Suspensions already slowly frozen at temperatures of -2° or lower, whether spontaneously or by inoculation with ice, could no longer be completely inactivated by subsequent rapid cooling to -196°C . Unfrozen suspensions initially above the freezing point or supercooled to -2°C or -8°C and then rapidly cooled to -196°C were inactivated. This protective action of previous slow freezing was most marked when the initial temperature of the frozen suspension was -15°C or lower, when it was -2°C protection was barely detected.

These observations indicate that the changes which are peculiar to rapid freezing alone and lead to complete inactivation take place during rapid transition from the liquid to the solid state, in a range of temperature lying between -15°C and the freezing point. Temperature measurements carried out in this range showed that suspensions were about equally infectious whether the temperature at their centers dropped from 0°C to -15°C in 30 minutes or in 1 minute, when the drop occurred in 12 seconds or less, the suspensions became innocuous.

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STUDIES ON THE NUTRITION OF HEMOPHILUS INFLUENZAE

I. THE RELATIONSHIP BETWEEN THE UTILIZATION OF COENZYME AND HEMIN AND THE REDUCTION OF NITRATE

By CHARLES L. HOAGLAND M.D. S. M. WARD HELENA GILDER, M.D. AND
ROBERT E. SHANK, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The specific requirement of influenza bacilli for a heat labile, vitamin like substance, in addition to hemin, or X factor, has been recognized for many years (1) Thjötta and Avery gave the name of V factor to this substance, and in a series of studies found it to have a wide distribution both in animal and in plant tissues (2) Rivers and Poole (3) showed that a convenient differential display of these two factors could be obtained by using filtered yeast extract as V factor and autoclaved blood as X factor. The identity of V factor with coenzyme was demonstrated by Lwoff and Lwoff in 1936 (4, 5). These authors suggested that the growth of *Hemophilus influenzae* be used as a quantitative measure of coenzyme by adding the test material to a medium devoid of this factor, but containing in excess all other substances necessary for growth of the microorganism. Kohn (6), Vilter, Vilter, and Spies (7), Pittman and Fraser (8), and others, have reported success in the use of the growth of *H. influenzae* and *H. parainfluenzae* as a measure of coenzyme in various materials. In all instances, turbidity has been employed as a criterion of growth of the organism, and indirectly as a measurement of coenzyme in a medium which they assumed to be adequate in all other respects for optimum growth of the bacilli. On the basis of this technique, studies on the coenzyme content in the blood of patients with pellagra (7) and of dogs with black tongue (9) have been reported, and observations on the *in vitro* and *in vivo* synthesis of coenzyme from nicotinic acid have been made (10).

During an attempt to confirm some of these observations and to study other phases of nicotinic acid metabolism, it became apparent that additional information concerning the nutritional requirements of *H. influenzae* and *H. parainfluenzae* was needed to establish the limitation of the technique and to make certain that conclusions reached were in all instances valid. It was soon evident that the use of turbidity as a measure of the growth of the influenza bacillus was subject to many limitations. In the first place, turbidity produced by growth of *H. influenzae* is rarely great, even under optimum conditions. Under conditions imposed by coenzyme restriction changes in turbidity resulting from changes in coenzyme concentration may be almost imperceptible, although marked differences in growth may be apparent when

plating and bacterial counts are performed. Secondly, unless test substances are quite clear and free from pigment they may contribute large errors to turbidimetric measurements, and at the present time no technique of extracting coenzyme has been devised which yields a completely clear extract and which at the same time insures complete extraction. Moreover, prolonged extraction and fractionation may well result in partial destruction of coenzyme.

- Successful microbiological methods have been developed recently for the quantitative determination of riboflavin (11), pantothenic acid (12), nicotinic acid and biotin (13). The emphasis placed on the determination of a stable metabolite, *i.e.*, lactic acid, in these methods, led us to search for a stable metabolic product of the influenza bacillus in the hope that the accurate measurement of such a substance could be substituted for the inaccurate measurement of turbidity as an index of growth and metabolic activity.

The ability of the influenza bacillus to reduce nitrate to nitrite has been noted consistently among its biochemical activities and was first studied in detail by Rivers (14) and Rivers and Kohn (15). In a survey of several metabolites produced by *H. influenzae* it has been found that the production of nitrite from nitrates can be used as a measurement of the metabolic activity of the bacilli and that the stability of this substance and the ease and great accuracy with which small amounts can be determined make it peculiarly applicable to a solution of the problems raised in a study of the growth requirements of this group of microorganisms. The use of this technique for the quantitative determination of coenzyme is being reported elsewhere. In this paper we wish to report studies showing the quantitative relationship between the utilization of coenzyme and hemin and the reduction of nitrate. Studies are also presented showing the influence of other substances in blood and tissue on the metabolic activity of influenza bacilli, as reflected in nitrate reduction, in the presence of optimum concentrations of hemin and coenzyme.

Materials and Methods

In an analysis of the part played by growth factors in the metabolism of *H. influenzae*, the experimental details are of great importance. Many of the equivocal and contradictory results in the literature can be explained only on the assumption that widely varying techniques were employed in attempts to elucidate similar phenomena. In some instances, large inocula have resulted in carrying over sufficient amounts of growth factors to last for several subsequent transfers. Another point contributing much confusion to an already complex picture, has been a lack of awareness of the small amounts of a given factor which may serve for the growth of *H. influenzae* in a medium in which the need for other required substances has been met. Moreover, failure to recognize the fact that X-factor is heat-labile under certain conditions may

have contributed great errors by permitting the assumption that this substance was present in excess and that increased growth of influenza bacilli was due to factors other than hemin in the materials added to the culture medium. And finally, quantitative differences in the requirement of various strains for hemin and coenzyme have not always been properly appreciated.

Source of Hemin or V Factor—Recrystallized, chemically pure hemin, obtained from the research laboratories of Eastman Kodak Company, was used. Similar results, however, were obtained with preparations of hemin made in our own and other laboratories. No important species differences have been observed.

Source of Coenzyme or V Factor—Preparations of coenzyme, or V factor, used in this study were for the most part made in our own laboratory from yeast by the method of Williamson and Green (16). Coenzyme prepared in this manner was shown to be active in the dehydrogenation of lactate, in the presence of lactic acid dehydrogenase and methylene blue, before being used in metabolic studies with *H. influenzae*. Further characterization of our coenzyme preparations was made by spectrophotometric studies in the ultraviolet range before and after reduction of the material with sodium formaldehyde sulfoxylate. In all instances a band at 2600 Å was observed with the material in the oxidized state, and a new band at 3450 Å appeared upon reduction with sodium formaldehyde sulfoxylate. The band at 3450 Å is characteristic of reduced coenzyme (17).

Since the requirement of influenza bacilli for factor V is met by coenzyme I or by coenzyme II, and since the band of reduced coenzyme in the ultraviolet at 3450 Å is the same in either case, no individual expression of coenzyme I or II in our preparations was attempted. These studies were made in collaboration with Dr. G. L. Lavin of the Spectroscopic Laboratory of The Rockefeller Institute.

Through the kindness of Dr. B. J. Jandorf of the Department of Biological Chemistry at Harvard University Medical School, we were able to secure a sample of coenzyme with a purity of 60 per cent as determined by comparison for activity with a standard sample which showed 93 per cent theoretical value for total phosphorus and 92 per cent for reduction with dithionite. In comparison with this standard, our less highly purified preparations were shown by the growth of *H. influenzae* to have a purity ranging from 32 to 43 per cent.

A solution containing approximately 50 micrograms of the partially purified coenzyme per cc. was sterilized by Berkefeld filtration. The concentration of coenzyme in the filtrate was determined by comparison of the ability of a suitably diluted aliquot to act as V factor with that of a standard desiccated sample of coenzyme of known purity which had been sterilized by treatment with ether. Following standardization, the filtrate was diluted to contain 4.5 micrograms of pure coenzyme per cc., and 0.5 cc. samples, accurately measured with a sterile Folin pipette, were delivered carefully into the bottoms of sterile test tubes. The material was dried *in vacuo* from the frozen state, the tubes plugged with sterile rubber stoppers and stored in a desiccator over CaCl_2 at 0°C. Coenzyme desiccated from the frozen state is completely and readily soluble and retains its full initial activity for several months. For use in the production of a standard growth curve with *H. influenzae* 10 cc. of

sterile distilled water were added to the dried coenzyme, care being exercised that none of the desiccated material escaped solution

Preparation of Media—A number of media proposed by various workers were tested. The one found best for our purposes was composed of 20 per cent Difco proteose-peptone, 0.6 per cent sodium chloride, and 0.2 per cent sodium or potassium nitrate. It was autoclaved for 20 minutes at 116°C. Except in those instances when the effect of hemin concentration on the growth of *H. influenzae* was being tested, 10 per cent autoclaved rabbit, sheep, or human blood was added to the medium after sterilization. No important differences among the bloods of various species could be determined. When blood was omitted from the medium, an autoclaved solution of hemin, as a source of X-factor, was added to make a final concentration of 0.01 mg. per cc.

Preparation of Inoculum—The strains of *H. influenzae* used in this study were obtained from a variety of sources, the primary requirement being that the organism selected should reduce nitrate readily and consistently. The final studies reported here were for the most part carried out with two strains of *H. influenzae* each originally isolated from a case of influenzal meningitis. Standard inocula were prepared in 5 cc. of a stock broth composed of 20 per cent proteose-peptone, 0.6 per cent sodium chloride, 0.01 mg. per cc. of autoclaved hemin, and 0.015 micrograms per cc. of purified coenzyme. No nitrate was added in order to prevent transfer of nitrite with the inoculum. Organisms carried on "chocolate" blood agar by weekly transfers were inoculated into a tube of stock broth and incubated overnight at 37°C. After three transfers in stock broth the organisms were ready for use. When 0.1 cc. of such a culture was inoculated into 10 cc. of a medium, no multiplication of the bacilli or production of nitrite occurred unless the medium contained coenzyme.

Measurement of Nitrite Production—For the measurement of nitrite the method proposed by Shinn (18) has been found very satisfactory. In this method the color produced by the diazotization of sulfanilamide and subsequent coupling of the diazo compound with *N*(1-naphthyl) ethylene diamine is dependent on and proportional to the nitrite present when the first two substances are added in excess. The original technique has been modified to some extent to permit its adaptation to samples containing peptone broth.—

0.1 cc. of culture, carefully measured, is added to 8.5 cc. of water, followed by 1 cc. of 0.2 per cent sulfanilamide, 0.2 cc. of 0.1 per cent *N*(1-naphthyl) ethylene diamine dihydrochloride, and 0.2 cc. of 6*N* hydrochloric acid. The color develops rapidly, is maximum in 5 minutes, and shows little change after 24 hours. The color intensity is read with a photoelectric colorimeter of the Evelyn type (19) with filter No. 520.

In the determination of nitrite, the symbols and terminology proposed by Evelyn for photoelectric colorimetry have been retained. If *G* refers to the galvanometer reading, *T* to transmittance of the solution, i.e., the ratio between the amounts of

light transmitted by the sample and the blank, then $T = \frac{G}{100}$, when the galvanometer

is adjusted to 100 with the tube containing the blank before the sample is read. For the color produced by coupling diazosulfanilamide to *N*(1-naphthyl) ethylene diamine, filter No. 520 has been found sufficiently selective to make the laws of Lambert

and Beer applicable. Hence, $C = \frac{1}{K} \times \log_{10} \times \frac{1}{T} = \frac{1}{K} \times \log \frac{100}{G} = 2 - \frac{\log G}{K}$

Therefore the concentration of chromogen, $C = \frac{L}{K}$ when $L = 2 - \log G$. Here K is a constant and L is referred to as "photometric density". In these studies it has been found more convenient to use the photometric density, rather than the concentration of chromogen, as an expression of the quantity of nitrite.

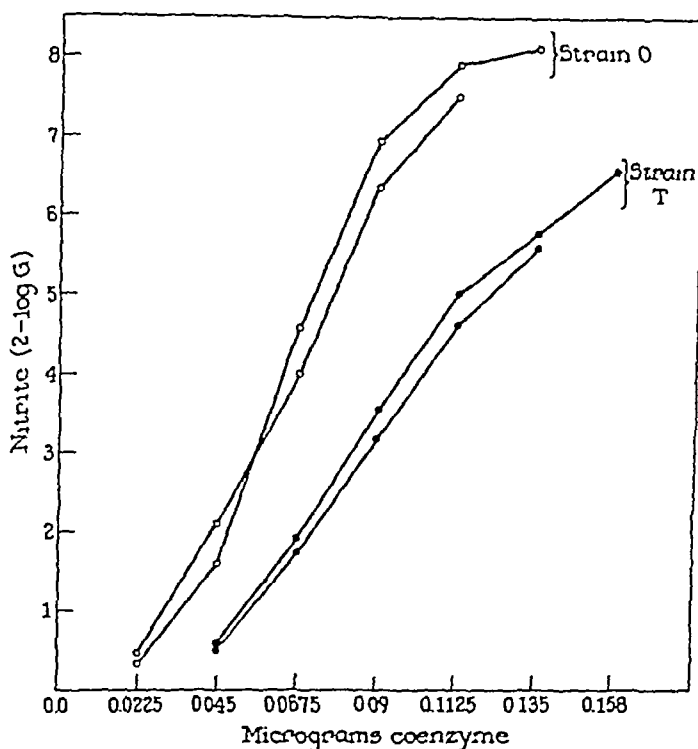
EXPERIMENTAL

In preliminary studies attempts were made to use ordinary bacteriological culture tubes for the growth of *H. influenzae*. It was soon apparent, however, that when such tubes were used the nitrite production was erratic and un dependable. With media deeper than 2 cm the amount of nitrite produced was not stoichiometrically related to coenzyme concentration. For this reason 50 cc. Erlenmeyer flasks were substituted for bacteriological culture tubes. With 50 cc. flasks containing 10 cc. of medium, consistent nitrite production, proportional to coenzyme concentration, has been achieved.

In all, five strains of *H. influenzae* have been used in this study. Although marked differences in the amount of nitrite produced were observed among different strains of influenza bacilli the total nitrite in each instance bore a stoichiometric relationship to the concentration of coenzyme in the medium when all other factors necessary for growth were present in excess. In Text fig. 1 it is seen that the nitrite produced by a given strain checked closely on two separate occasions. The amount of nitrite formed by strain T, however, was considerably less than that formed by O, which indicates that a given strain of *H. influenzae* may be characterized to some extent by the effectiveness with which it reduces nitrate.

Six concentrations of sterile coenzyme, 0.0, 0.0225, 0.045, 0.0675, 0.09, and 0.1125 micrograms, respectively were added to 9 cc. of the media containing 1 mg. per cent hemin as described above and sufficient sterile water added to bring the volume to 10 cc. the series was set up in triplicate. The flasks were then inoculated with 0.1 cc. of a standard culture of *H. influenzae* previously described, and incubated for 48 hours at 37°C. 0.1 cc. of culture was then added to 8.5 cc. of water and the nitrite determined as outlined above. Since the range of nitrite concentration which can be determined by diazotization and production of color by coupling is fairly narrow and the amount of nitrite produced by *H. influenzae* is relatively great for small increments of coenzyme, it has been found best, in most instances, to limit the amount of culture taken for nitrite analysis to 0.1 cc. However, with certain strains of *H. influenzae* which reduce nitrates less effectively, 0.2 to 0.5 cc. of the culture media may be required for an accurate determination. When 1 cc. or less of the medium is taken for analysis, turbidity of the culture or test material does not interfere significantly with the photoelectric determination of the color intensity.

Heat Stability of the X-Factor Redefined—It has been stated by various workers from time to time that X-factor in certain materials may be partially destroyed through autoclaving (20). That this destruction may be very great, even to the extent of rendering media deficient in this substance, has been brought out by quantitative studies on a comparison between the extent of growth of *H. influenzae* in media to which hemin has been added before



TEXT-FIG 1 Relationship between the concentration of coenzyme and the production of nitrite by *H. influenzae* in a medium containing an excess of hemin

autoclaving and autoclaved media to which X-factor, separately autoclaved, has been supplied. X-factor concentrations which, if separately autoclaved, would have been sufficient to permit maximum growth of *H. influenzae*, may, following autoclaving of X-factor and media together, fail to support visible growth of the organism. In quantitative studies on the metabolism of coenzyme by *H. influenzae* an excess of the X-factor is absolutely necessary, otherwise the growth stimulus from materials containing coenzyme may be due in part to hemin which these test substances contain.

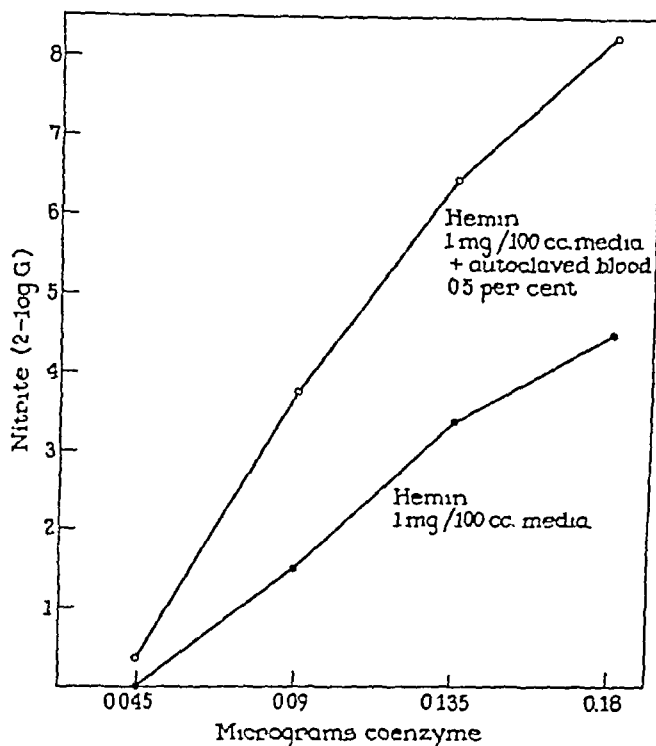
The extent to which X-factor may be destroyed by being autoclaved with the

basal medium was shown by a comparison of the growth of *H influenzae*, in the presence of increments of coenzyme and an experimentally determined excess of autoclaved hemin, with the growth of *H influenzae* under identical conditions, except that the hemin was autoclaved with the basal media. The growth in the second series was markedly decreased. That the decreased growth was caused by a lack of sufficient X factor was shown by the fact that the basal media were again rendered adequate for optimum growth by the addition of hemin which had been autoclaved separately. In a number of reported studies in which the growth of *H influenzae* has been used as a measure of coenzyme concentration, these factors have not been controlled. Consequently it is possible that any observed stimulation was due to additional X factor added with the coenzyme test material, and not to the coenzyme, or V factor, alone. This criticism may be particularly leveled at those reports in which a proteose-peptone medium, without the addition of sufficient X factor, has been used in the quantitative assay of coenzyme. For example, media prepared according to Vilter, Vilter, and Spies (7) has, in our hands, consistently failed to grow *H influenzae* when highly purified coenzyme preparations were added to supply V factor. Growth is obtained, however, when hemin is added subsequently, or when a blood extract, prepared according to the technique of these workers, is used as a coenzyme test substance. In the latter instance sufficient hemin is carried over in the acid extract to permit growth in the presence of the coenzyme which the extract contains. However, when test substances which contain little hemin are being assayed, the factor limiting growth of *H influenzae* is no longer due to coenzyme, but to the variable quantity of hemin which may be present.

Additional Factors in Blood Which Stimulate the Growth of H influenzae — The development of a more exact quantitative method for the determination of the metabolic activity of *H influenzae* has made possible a study of the effect of various nutrient substances on the growth of this group of organisms. That there are additional factors in blood, other than hemin and coenzyme, which enhance the growth of *H influenzae* is evident in the data portrayed in Text fig 2.

To duplicate flasks of 10 cc. of basal media were added an experimentally determined excess of hemin (1 mg per 100 cc.) and increments of coenzyme from 0.0 to 0.1125 micrograms. These were inoculated with a standard strain of *H influenzae*, prepared in the manner previously described and incubated for 48 hours at 37°C. Nitrite determinations were made and the results have been plotted in Text-fig 2 against increments of coenzyme. Another series of flasks containing 0.5 per cent autoclaved rabbit blood, in addition to the constituents of the basal medium, were set up, and hemin to make a final concentration of 1.0 mg per cent was added. Increments of coenzyme, as described in the first series, were added and the flasks inoculated with 0.1 cc. of a standard culture of *H influenzae* and incubated for 48 hours.

In Text-fig 2 the results of nitrite reduction vs increments of coenzyme are compared with the series in which hemin, but no blood, had been added to the proteose-peptone medium. In all instances a definite and measurable increase in nitrate reduction was noted when blood was added to the basal medium. The addition of more than 0.5 per cent of blood, however, failed to increase stimulation. It would appear, therefore, on the basis of these studies, that



TEXT-FIG 2 Production of nitrite by *H influenzae* in a medium containing increments of coenzyme and an excess of hemin, and with and without the addition of 0.5 per cent blood.

additional factors, either organic, inorganic, or both, in addition to hemin and coenzyme, may enhance the growth of *H influenzae* in the basal medium, and that these are maximally supplied by autoclaved blood when it is added in a concentration of 0.5 per cent.

Influence of Known Growth Factors on the Metabolism and Growth of H influenzae—In order to use the metabolic activity of the influenza bacillus as an index of coenzyme concentration it is important to know what effect may be expected from an increased concentration of other known growth factors

which may be added in varying amounts as contaminants in coenzyme test materials. In Table I are listed a large number of substances which have been added to blood-enriched, proteose-peptone media inoculated with *H. influenzae*. In no instance has any significant difference been noted in the amount of nitrite produced between the control cultures grown in basal media with added hemin and coenzyme and those cultures containing large amounts of added growth factors in addition to the constituents of the control media. Moreover, a number of amino acids, added separately and in combination, produced

TABLE I

Effect of Added Nutrient Materials on the Growth and Metabolism of H. influenzae in a Basal Medium Containing an Excess of Hemin and Coenzyme

[Substance	Concen- tration per cc. of medium	Effect on growth as reflected in nitrite production	Substance	Concen- tration per cc. of medium	Effect on growth as reflected in nitrite production
	micrograms			milligrams	
Pantothenic acid	0.05	None	β -Alanine	0.5	None
Nicotinic acid	0.05		Inositol	0.1	
Nicotinamide	0.05		Ascorbic acid	0.05	
Pyridoxin	0.05	"	Cytochrome c	0.05	"
Thiamin	0.05		Ergothioneine	0.025	
Biotin	0.002		Tryptophane	0.05	
Choline	0.05	"	Arginine	0.05	"
Folic acid	0.05		Amino acid mixture		
Paraminobenzoic acid	0.05		Methionine	0.06	
Riboflavin	0.05		Tryptophane	0.06	
Riboflavin adenine dinu- cleotide	0.05		Histidine	0.06	
Riboflavin phosphate	0.05		Leucine	0.20	
Thiamin pyrophosphate	0.05		Glycine	0.02	
			Glutamic acid	0.04	
			Arginine	0.06	
				0.50	

no significant alteration of growth or metabolic activity in this medium. These are likewise recorded in Table I.

The fact that influenza bacilli do not use nicotinic acid as such, but require its physiologically active form, coenzyme, led us to try the activated forms of riboflavin and thiamin, i.e., riboflavin adenine-dinucleotide, riboflavin phosphate, and thiamin pyrophosphate. Riboflavin adenine-dinucleotide was prepared from yeast by the method of Warburg and Christian (21), riboflavin phosphate was synthesized by the method of Kuhn, Rudy, and Weygand (22), and thiamin pyrophosphate was synthesized by the method of Weijlard and Tauber (23). No stimulation by any of these substances to the growth or metabolic activity of *H. influenzae* could be detected when they were added to

the blood-enriched proteose-peptone medium. It cannot be concluded from these results, however, that these substances are not required by the influenza bacillus, since the heat stability of riboflavin adenine-dinucleotide, riboflavin phosphate, and thiamin pyrophosphate, unlike coenzyme, would insure a certain concentration of these catalysts in the blood-enriched basal medium. It can only be stated that if these factors are active in promoting the growth of *H. influenzae* they are present in excess in the basal medium, since added amounts produced no detectable stimulus to growth or metabolic activity over that observed in control cultures.

DISCUSSION

In the foregoing studies we have attempted to define more carefully the conditions under which the growth and consequent metabolic activity of *H. influenzae* may be correlated with the total coenzyme, or V-factor, content of the medium. It is obvious that unless all specific requirements for the growth of the influenza bacillus are fully met, a quantitative relationship between metabolic activity and coenzyme content of the medium cannot be expected. Moreover, it is essential that substances not specifically required for growth, but which influence its extent, must likewise be supplied in excess, otherwise they may have a marked stimulating effect on the degree of growth when added with materials which are being tested for coenzyme activity.

The difficulties previously encountered in the measurement of the growth of *H. influenzae* have been overcome by a technique which substitutes for the measurement of turbidity the quantitative determination of the nitrite produced by these organisms in a medium to which nitrate has been added. The production of nitrite has been shown to be a consistent measure of the metabolic activity of the influenza bacillus, and, when other requirements of the organism are met in excess, bears a consistent relationship to the concentration of coenzyme in the medium. In these studies the nitrate reduction technique has been used in an attempt to define the optimum growth requirements of *H. influenzae*, which, in turn, are to form the basis of further studies on the nutrition and metabolism of this organism.

When optimum amounts of hemin and coenzyme are present, additional requirements and growth-modifying factors appear to be supplied in excess when 0.5 per cent blood is added to a proteose-peptone medium. This is shown by the fact that no appreciable alteration in growth or metabolic activity of *H. influenzae* can be demonstrated when an increased concentration of various factors which are known to enhance bacterial growth are added to a blood-enriched proteose-peptone medium. It is essential in those instances in which hemin is added as the main source of X-factor that the hemin be autoclaved separately, otherwise sufficient destruction may result to render the medium deficient in this substance.

From these studies it appears that extensive information concerning factors affecting the growth and metabolism of a test organism must be had before it may be used accurately as a specific biological reagent for microbiological assay. Not only is it essential to know the specific factors required for growth of the organism and the optimum concentration of these factors, but information must likewise be had concerning those substances which, although not essential for the organism, may, nevertheless, affect the extent of its growth.

CONCLUSIONS

The metabolic activity of *H. influenzae* can be followed quantitatively by measurement of the nitrite produced in a medium containing 0.2 per cent potassium or sodium nitrate.

When X factor, or hemin, and other specific substances required for the optimum growth of *H. influenzae*, are present in excess, the nitrite produced by this organism is quantitatively related to the concentration of V-factor, or total coenzyme. This quantitative relationship has been demonstrated for five strains of *H. influenzae*.

It has been shown that various media, which in the past have been used for the determination of coenzyme by growth of *H. influenzae*, have in many instances been deficient in X factor and that this substance rather than coenzyme has been the specific factor limiting growth.

When 0.5 per cent blood is added to a basal proteose-peptone medium the specific requirements for optimum growth and metabolic activity of *H. influenzae*, other than coenzyme, are met, and a large number of specific biocatalysts and nutritive substances added to this medium are without effect in stimulating further growth.

The foregoing studies have formed the basis for a quantitative method for the determination of total coenzyme in blood and tissue. This method is being described elsewhere.

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complicated by the fact that endemic pneumonia, which is a separate entity, may be apparent at any age but particularly from the 6th month on. Rat catarrh *per se* is probably never fatal, the few observed deaths have been restricted to old rats which were also infected with endemic pneumonia.

The microscopic examination of exudate from the 3 hosts infected either by nasal instillation or contact regularly shows small spherical or slightly elongated Gram-negative particles tentatively called coccobacilliform bodies. These bodies have been isolated in a pure state, propagated in tissue cultures, and established beyond a reasonable doubt as the etiological factors of the 3 diseases.

While the present work was in progress it was found that the specific agent of mouse catarrh produced a demonstrable reaction in the mouse on extranasal administration. 60 per cent of 15 mice injected in the foot pad with either exudates or tissue culture suspensions showed an arthritic response limited to the injected leg and indicated by swelling of the knee joint, the leg from the joint downward, or the toes. The incubation period varied from 7 to 14 days. The course of the reaction was also variable, occasionally resulting in permanent injury with stiffness of the leg, but more often subsiding after several weeks. Films of joint fluid regularly showed poly nuclear leucocytes but no coccobacilliform bodies. Colonies of pleuropneumonia-like organisms were not demonstrable through the 10th day on 30 per cent horse serum agar plates inoculated with fluid. Coccobacilliform bodies were recovered from one fluid on inoculation in tissue cultures. This fluid produced all the features of infectious catarrh on nasal instillation in mice but was innocuous on foot pad injection, as were several other fluids which were injected by this route. The arthritic reaction has always been restricted to the injected leg and has never been observed in mice infected by way of the upper respiratory tract.

A less conspicuous local reaction was also observed in rats injected in the foot pad with homologous exudates. The morbidity rate in 10 rats was 50 per cent, the reaction being limited to a bulbous swelling of the toes or a thickening of the leg from the knee joint downward. Fluid removed by aspiration showed polynuclear leucocytes but no coccobacilliform bodies and was sterile on 30 per cent horse serum agar plates.

Observations were made on the susceptibility of each host, namely, chickens, mice, and rats, to the 3 forms of infectious catarrh. The respective causal agents were introduced directly by nasal instillation and foot pad injection of exudate and tissue culture suspensions, and indirectly by contact exposure.

The Insusceptibility of Mice and Rats to Infectious Catarrh of Chickens

Exudate suspensions infective for chickens failed to produce any detectable reaction in mice or rats on nasal instillation or foot pad injection.

The mice used throughout these experiments varied in weight from 15 to 20 gm., the rats were 2 to 3 months old, as were also the chickens which were chiefly Rhode Island Reds. The 3 groups of animals were removed at random from stocks reared at The Rockefeller Institute in Princeton, New Jersey, and known to be free from the

particular infections under consideration 2 lots of exudate aspirated from the nasal passages and sinuses of infected birds (passages 65 to 67) and suspended in saline were used in the following tests. 5 of 6 chickens infected by injection through the palatine cleft showed a nasal discharge after 16 to 18 days. In one bird inflammation of the upper air passages was apparent only at autopsy. Coccobacilliiform bodies were demonstrable microscopically in exudate films from all 6 chickens. 10 mice and 5 rats were etherized and injected intranasally by introducing 8 to 10 drops of the exudate suspensions in the nostrils. 6 mice and 6 rats were injected in one foot pad with approximately 0.05 to 0.1 cc. of the same material. All of these animals remained normal during an observation period of 4 to 5 weeks and were normal throughout at autopsy.

The Susceptibility of Rats and Chickens to Infectious Catarrh of the Mouse

The Reaction of the Rat to Exudate Injected Intranasally—Infectious catarrh of the mouse was established in rats on the initial nasal instillation of middle ear exudate from experimentally infected animals and was maintained by passage. The morbidity rate in 27 rats used in 4 passages was 70 per cent, the incidence of otitis and rhinitis being 62 and 55 per cent, respectively. Coccobacilliiform bodies were demonstrable microscopically in all exudates. Unlike the reaction in mice, however, there was no involvement of the lung, at least through the 18th week after nasal instillation, and no mortality. Exudate from the 4th rat passage on transfer to mice by the nasal route produced the customary pneumonia in addition to otitis and rhinitis.

The rats in the first 3 passages were killed and autopsied 4 weeks after injection while those in the 4th passage were held under observation for 18 weeks. The inoculum in each of the passages was middle ear exudate suspended in saline. Prior to autopsy all of the rats were normal save for snuffling, which was often observed by the 3rd week. 4 of the 5 mice injected with exudate from the 4th rat passage died 2 to 6 months later the lungs being extensively involved at autopsy. One mouse was killed during the 5th month and also showed an extensive pneumonia in addition to otitis and rhinitis.

The Reaction of the Rat to Exudate Injected in the Foot Pad—Exudate from infected mice was also active on foot pad injection in rats. The reaction was slight, characterized by swelling of the knee joint or toes of the inoculated leg, and the rate low, 33 per cent in 10 animals.

The arthritic reaction was first observed during the 3rd week after foot pad injection. In one rat it was limited to the knee joint and in 2 animals to a single toe. At autopsy a small amount of fluid containing polynuclear leucocytes was removed from the affected areas on incision. There was no involvement of the respiratory tract or middle ears in any of the rats.

The Reaction of the Rat to Tissue Cultures of the Coccobacilliiform Bodies—A tissue culture of the mouse catarrh agent isolated from the middle ear of an

infected animal behaved essentially the same in rats as did exudate. The observations on the activity of this culture in rats and in mice are summarized in Table I.

The inoculum used in the above tests was prepared from the 6th transfer of a 72 hour tissue culture of the coccobacilliform bodies, the tissue being ground and suspended in 2.0 cc. of the supernatant.

The rats and mice in the nasal series showed snuffling and chattering, respectively, during the 3rd week after injection and were killed for examination at the end of the 4th week.

The foot pad injection produced an inflammatory reaction in 2 of the 5 rats, limited in one animal to the knee joint and in the other to a single toe. In the second of these rats there was a definite unilateral otitis. Exudate films

TABLE I
Behavior of the Coccobacilliform Bodies of Mouse Catarrh in Rats and Mice

Animal injected	No. injected	Route of injection	Cases of			
			Pneumonia	Otitis	Rhinitis	Arthritis
Rats	5	Nasal	0	4	4	0
Mice	5	"	5	4	4	0
Rats	5	Foot pad	0	1*	0	2
Mice	5	" "	0	0	0	5

* Presumably a sporadic case unrelated to the route of injection.

showed numerous polynuclear leucocytes but no coccobacilliform bodies. A non-hemolytic streptococcus was isolated in pure culture from an inoculated blood agar plate. It is probable that the middle ear involvement in this rat was representative of the sporadic type of otitis which is occasionally encountered in our rat colony.

All of the 5 mice injected in the foot pad showed an arthritic reaction limited to the knee joint, apparent after 9 to 15 days. On incision fluid containing leucocytes was removed, a pure tissue culture of the coccobacilliform bodies being isolated from one specimen. The respiratory tract and middle ears were normal in all of the animals.

The Reaction of the Rat on Exposure to Infected Rats and Mice—Limited observations on the communicability of mouse catarrh indicated that it was transmissible by direct contact from infected to normal rats and from infected mice to rats. The morbidity rate of 10 rats thus exposed was 50 per cent, the 5 infected animals showing no involvement of the lung. Coccobacilliform bodies were demonstrable in middle ear exudate which on nasal instillation in mice again produced a pneumonia.

In the rat to rat contact experiments 5 normal animals were placed in the same cage with one rat previously infected by the nasal injection of mouse exudate, and exposed for 13 weeks. At autopsy 4 of the 6 rats, including the originally infected one showed rhinitis and otitis, the specific agent being demonstrable in all exudate films. The lungs were uniformly normal. Middle ear exudate from 2 of the rats infected by contact was injected intranasally in 5 mice. Chattering was first noted during the 3rd week and at autopsy during the 6th week all of the mice showed pneumonia, rhinitis, and otitis.

In the mouse to rat contact experiment 5 normal rats were placed in the same cage with 4 mice infected earlier via the nasal route. One mouse died 2 weeks later, 2 were killed after 5 weeks, and the 4th after 8 weeks, at which time the rats were also killed. 3 of the mice were autopsied with the usual findings. 3 of the rats were normal at autopsy, while 2 showed a unilateral otitis with a protrudent tympanic membrane. Coccobacilli-form bodies were present in films. The nasal passages and lungs of these 2 rats were normal.

The Reaction of the Chicken to Exudate Injected into the Nasal Passages—Chickens were injected only with exudate from infected mice, the inoculum being introduced by way of the palatine cleft. The specific agent failed to establish itself on the mucous surfaces of the upper air passages. In 6 birds held under observation for 4 to 5 weeks there was no discharge through the nares during life, and at autopsy the respiratory tract and communicating sinuses were normal. Coccobacilli-form bodies were not demonstrable microscopically in nasal washings.

The chickens in this experiment were injected in 2 groups of 3 with exudate from the middle ears of 7 infected mice. During the period of observation they were kept in single cages and examined daily. The exudate suspensions used in these tests were likewise injected in mice and produced the characteristic manifestations of infectious catarrh.

The Susceptibility of Mice and Chickens to Infectious Catarrh of the Rat

The Reaction of the Mouse to Exudate Injected Intranasally—Infectious catarrh of the rat was transmitted to mice by the nasal instillation of middle ear exudate and maintained by passage. The morbidity rate in 10 mice which received exudate directly from infected rats was 100 per cent, all of the animals showing both otitis and rhinitis at autopsy and 8 of them pneumonia. These mice were killed 4 to 5 weeks after injection. In 2 successive passages 22 additional mice were used and the period of observation was extended. 20 of these mice (90 per cent) died at intervals ranging from 11 to 206 days. Coccobacilli-form bodies were demonstrable microscopically in all exudates from the middle ears and nasal passages.

Two groups of 5 mice were used in the first transmission experiment and were injected with exudate removed from rats of the 40th and 41st passage series, respec-

tively. Chattering was first observed 19 days later. The 2 mouse passages were made with exudate from a single animal of the preceding transfer. 2 of these mice were killed during the 1st and 6th months after injection, the lungs, middle ears, and nasal passages being involved. Most of the mice that died were sufficiently well preserved to be examined and showed an advanced pneumonia at autopsy.

The Reaction of the Mouse to Exudate Injected in the Foot Pad—Exudate from infected rats produced a local inflammation in 7 of 15 mice (46 per cent) following injection in the foot pad. None of the mice infected by this route showed any involvement of the respiratory tract or middle ears.

The mice in this experiment were injected in 3 groups of 5 with saline suspensions of middle ear exudate from infected rats of the 38th, 40th, and 41st passages, respectively. The incubation period of the arthritic reaction was 13 to 19 days. It was characterized by edema which spread along the leg from the knee joint downward in 3 mice, also involving the toes in one mouse, and limited to the joint in 4 animals. 3 of the mice were killed shortly after the appearance of symptoms and fluid containing leucocytes was removed from the affected joint.

The Reaction of the Mouse to Tissue Cultures of the Coccobacilliform Bodies—The coccobacilliform bodies of rat catarrh grown in tissue culture were also established in mice on initial nasal instillation and maintained for 6 successive transfers. The morbidity rate in the first 3 passages was 86 per cent, which compares favorably with the rate following the injection of exudate. The reaction was noticeably less extensive, however, being limited largely to the middle ears. None of the 15 mice showed any involvement of the lung at autopsy, and in only 4 (26 per cent) was there any involvement of the nasal passages. A unilateral or bilateral otitis was regularly observed in the 13 infected animals. Pneumonia was first noted in mice of the 4th passage and continued through the 6th, when the series was discontinued, the incidence in the 15 mice being 62 per cent. The specific agent was observed microscopically in all exudate films.

A single experiment was carried out on the behavior of the rat catarrh agent in pure culture following foot pad injection in mice. After incubation periods of 18 and 21 days, respectively, 2 of 5 mice showed an arthritic reaction which was restricted to the knee joint.

The culture of the rat catarrh agent used in these experiments was isolated from the middle ear exudate of 2 rats in the 40th passage series. The inoculum was prepared from a 48 hour growth of the 10th tissue culture transfer. It showed numerous coccobacilliform bodies microscopically but was otherwise bacteriologically sterile. The subsequent passages were made with middle ear exudate from one or more mice, the interval of transfer varying from 1 to 3 months. The rates of otitis and rhinitis could only be approximated in the mice of the last 3 passages because of extensive mutilation after death. The otitis rate appeared to be normal and the rhinitis rate low.

The Reaction of the Mouse on Exposure to Infected Mice and Rats—Rat catarrh established in mice by the nasal instillation of exudate was transmissible from them to normal mice by direct contact, the morbidity rate of 15 mice thus exposed being 100 per cent. The mortality rate of 10 mice in this series, which were held for periods longer than a month, was 90 per cent, one infected animal surviving for a year. The only selected rats available at that time for determining the effect of mouse passage on the infectivity of exudate for the rat were injected with material from this animal, 4 of the 5 rats showing catarrhal manifestations on subsequent autopsy.

Limited observations on infected rats also indicated that the disease was transmissible from them to exposed mice. Compared with the results of the mouse to mouse contact test, the morbidity and mortality rates for 8 mice were considerably reduced, being 60 and 20 per cent, respectively. Coccobacilliform bodies were regularly observed in exudate films from the infected mice of both contact groups.

In the first contact experiment 5 normal mice were placed in the same cage with one infected mouse and killed after an interval of approximately a month. At autopsy each of the 5 mice showed a bilateral or unilateral otitis, 2 rhinitis but none of them pneumonia. In 2 other experiments the mice were similarly exposed but the period of observation was extended. 9 of 10 mice died 3 during the 10th to 13th week, and 6 during the 23rd to 43rd week. One mouse was alive on the 385th day after exposure and was then killed. At autopsy there was an inflammation of both middle ears and the nasal passages, together with an extensive pneumonia involving 4 lobes of the lung. 5 rats were injected intranasally with middle ear exudate from this mouse and were killed 5 weeks later. At autopsy 4 of the rats showed an inflammation of the nasal passages coccobacilliform bodies being present in films, but not of the middle ears. This condition is not uncommon when exudate from an animal that has been infected for a long period is used. One of the 4 infected rats also showed an involvement of one lobe of the lung the specific agent again being demonstrable. This rat was the only one in the entire series in which there was any macroscopic involvement of the lung. One rat in this group was uninfected, all loci being normal.

In the rat to mouse contact test 8 normal mice were placed in the same cage with 4 previously infected rats and contact was maintained for 14 days. One mouse was found dead on the same day that the rats were removed from the cage and showed typical catarrhal manifestations at autopsy. 4 of the 7 surviving mice, which were killed 9 weeks later, were infected while 3 were normal.

The Reaction of the Chicken to Exudate Injected Intranasally—Irregular results were obtained with chickens following the nasal injection of middle ear exudate from infected rats. There was an indication that the coccobacilliform bodies were established in the upper respiratory tract, but their survival was brief since they were not demonstrable after the 2nd passage. Owing to the lack of normal selected rats for test purposes and for maintenance of the disease in the rat, it has not been possible as yet to repeat this experiment.

The exudate used in the initial injection, made via the cleft palate, was removed from 2 rats of the 47th passage series. The chickens in this passage and in the 2 subsequent ones were kept under quarantine in single cages. They showed no symptoms during the period of observation which varied from 4 to 5 weeks. In 2 of 3 birds in the 1st passage there was an inflammation of the sinuses at autopsy. In the 2nd passage there was an inflammation of the nasal passages and the trachea in one bird. Coccobacilli-form bodies together with numerous leucocytes were demonstrable in all exudates. In the 3 birds of the 3rd passage there was no apparent involvement of the upper respiratory tract and coccobacilli-form bodies were not seen in films of nasal washings. The exudate used in this passage was also injected intranasally in 5 mice. At autopsy 4 weeks later the lungs, nasal passages, and middle ears were normal in all of these animals.

The Behavior of Three Additional Strains of Rat Catarrh

During the summer of 1941 the fertility of our selected rat colony, which had been reasonably constant since it was established in 1931, began to decline and by fall the population of young rats was reduced to a single litter. Coincident with this decline was a persistent exanthem particularly noticeable about the head. With the addition of green food, which had been lacking in the diet for a period of years, breeding was reestablished and the skin lesion largely disappeared.³ For a number of months, however, there were no rats available for experimental purposes and accordingly an attempt was made to locate normal animals from other sources.

Sample rats were obtained from two large commercial colonies and examined on the day of arrival or shortly after. 9 of 11 rats from the first dealer and 6 of 6 from the second showed unmistakable evidence of infectious catarrh at autopsy. These animals were supposedly representative of their respective colonies which supply large numbers of rats to laboratories throughout the country. Inspection of the colony operated by the second dealer revealed an appalling amount of snuffling in the breeding stock. This symptom is indicative of infection but is not strictly pathognomic of rat catarrh. A generalized transmission of this and other communicable diseases was assured in this colony by the practice of periodic rotation of breeders. Inquiries were also made of two other dealers who operate smaller colonies as to the condition of their rats. Both admitted that snuffling was noted in their breeding stock.

About this time a group of young rats was brought to us from a colony maintained for experimental purposes. These animals had been maintained on a balanced diet but showed unexplained weight losses. At autopsy a diagnosis of infectious catarrh was made in 9 of 12 animals.

Infectious catarrh was established in mice following the initial nasal instillation of middle ear exudate from each of the three groups of rats obtained from outside sources. The morbidity rate of 15 mice used in the three tests was 100 per cent. The incidence of pneumonia, 40 per cent, was somewhat low.

³ We are indebted to Mr. E. Raymond Ring for his supervision of the selected rat colony during this period.

in comparison to that associated with the local strain of the disease, but none of the mice was held longer than 2 months and most of them were killed 4 weeks after injection. Coccobacilliform bodies were observed in films of exudate from all of the mice and also from the rats which supplied the material for injection

DISCUSSION

The results of the preceding tests indicate that the relation between avian and rodent infectious catarrh is not sufficiently close to permit an unrestricted intertransmission of the respective coccobacilliform bodies. Homologous exudates infective for chickens failed to produce any reaction in mice or rats. Mouse exudates were also inactive in the chicken, but rat exudates elicited a definite though transient response on nasal instillation. Repetition and extension of the latter experiments have not been possible as yet because of the scarcity of disease-free rats, but are indicated in view of the possible transfer of rat catarrh to chickens by wild rats.

A close relation between the two rodent forms of infectious catarrh is, however, clearly established. Reciprocal transmission of the respective coccobacilliform bodies was demonstrable in both the mouse and the rat by nasal instillation, foot pad infection, and direct contact. Actual identity of the two diseases is suggested but not confirmed, cross agglutination and protection tests indicative of their precise relation not being applicable at present. If the anomalous results obtained in the chicken are valid, they are not necessarily contradictory of identity as they might be referable only to strain differences. In view of the close agreement between infectious catarrh of the mouse and the rat in respect to intercommunicability, the issue of identity is of little added significance.

The dissimilarity between the two rodent forms of the disease regarding the regular development of pneumonia with high mortality in the mouse and its occasional occurrence without death in the rat, is apparently referable to the respective animal host and not to actual differences in the coccobacilliform bodies. Infectious catarrh of the rat on transfer to the mouse was attended by a fatal pneumonia, whereas the mouse form rarely provoked pneumonia in the rat and never resulted in death. It is probable that the different response of the mouse and the rat to the same agent is indicative of differences in natural resistance, but there is a possibility that it may be related in some way to the endemic pneumonia which occurs so commonly in the rat.

Fowl coryza is a disease of recognized economic significance in the poultry industry. The importance of the rat and the mouse in experimental biology is, we believe, sufficient justification for also regarding the two forms of rodent catarrh as more than merely laboratory curiosities.

The rat is not widely used as a laboratory animal in pathology, but is in

physiology, particularly in the field of nutrition. It is probable that the spread of infectious catarrh in the rat is as general as endemic pneumonia and is more likely to be encountered since it affects animals of all ages, whereas the pneumonia is largely confined to older animals. It is also probable that experiments in nutrition are often made with naturally infected rats. The only symptoms of the disease are snuffling, which is not always apparent particularly in the late stages of infection, and twisting, which is generally of rare occurrence. It is a common practice of dealers to sell rats which appear normal, and of investigators to use them with no actual knowledge of the incidence of infectious catarrh, which can be gained only by periodic autopsy with examination of the middle ears. The use of abnormal animals engendered by this practice introduces a possible source of error which should not be overlooked.

The mouse is extensively used in pathology and frequently for the study of respiratory diseases of other animals and man. Infectious catarrh is obviously a hazard in such work, particularly if successive passages are made. In addition to acting as contaminants, the coccobacilliform bodies, by altering the mucous surfaces of the respiratory tract, might also retard or enhance the activity of other agents introduced by nasal instillation. As in the rat, the external appearance of the mouse is of little aid in diagnosis since symptoms may be lacking in the early development of the disease. At present it is likely that infectious catarrh is not as widely spread in the mouse as in the rat. That it may become so is suggested by the fact that many dealers who handle rats have also established colonies of Swiss mice, thereby introducing the risk of transmission by contact.

SUMMARY

Infectious catarrh of chickens (fowl coryza of slow onset) was not transmissible to mice or rats by nasal instillation of the specific coccobacilliform bodies. Exudates were also inactive in both rodents on foot pad injection.

The infectious catarrhs of the mouse and the rat were reciprocally transmissible by the nasal injection of exudates or tissue cultures of the respective coccobacilliform bodies and by direct contact. Exudates and cultures also produced an arthritic reaction in both hosts on foot pad injection.

The coccobacilliform bodies of mouse catarrh were innocuous in chickens on nasal instillation, whereas those of rat catarrh were established locally but were maintained for only two passages.

In the opposite host each of the two rodent forms of infectious catarrh reproduced the typical features of the naturally acquired disease, a highly fatal pneumonia being characteristic of the mouse but not of the rat.

EXPERIMENTAL INFECTION OF THE CHICK EMBRYO WITH THE VIRUS OF PSEUDORABIES

By FREDERIK B. BANG, M.D.

*(From the Department of Animal and Plant Pathology of The Rockefeller Institute for
Medical Research, Princeton, New Jersey)*

PLATES 15 AND 16

(Received for publication, May 22 1942)

Experimental infection of the chick embryo with certain bacteria (1-6) and viruses (7-11) has yielded new information on the host-parasite relationship of these agents. For example, when inoculated on the chorioallantoic membrane, vaccinia virus spreads to the embryo and produces a generalized pock disease (7). Following intra-amniotic injection the virus of human influenza causes a destruction of the embryo lungs (8). Swine influenza virus does the same following membranous inoculation (9), and rabies virus produces extensive destruction of the embryo central nervous system (10). In this last, the general pattern of infection simulates that of the natural disease; many Negri bodies are found in all types of nerve cells, neuronophagia and perivascular infiltration are not observed.

The virus of pseudorabies readily infects the chorioallantoic membrane of chick embryos and produces pocks which may coalesce to form large ulcers (12, 13). Since pseudorabies causes a rapidly destructive encephalitis in a wide variety of animals (14, 15), a selective effect on the central nervous system of the chick embryo is to be expected. In contrast to the virus of herpes which has no apparent neurotropic tendencies in the chick embryo (11), all four of the strains of pseudorabies here studied have a marked neurotropism.

EXPERIMENTAL

The following four strains of virus were kindly furnished by Dr. R. E. Shope: (1) A Hungarian strain sent to Dr. Shope by Dr. Aujeszky in 1931. (2) The Iowa A strain isolated in 1930 by Shope (15) from cattle with 'mad itch'. Both have since been put through over 50 serial rabbit brain passages and are here arbitrarily considered as 'fixed' strains. (3) The Iowa B strain isolated by Shope (16) from a pooled sample of two cow brains from Johnson County, Iowa. (4) The swine strain isolated by Dr. J. D. Ray (17) from naturally infected pigs in Nebraska. The last two had had respectively 15 and 5 intracerebral passages. All were again identified after chick embryo passage by one or more of the following characteristics: ability to produce a rapidly fatal (24 hours) encephalitis in the rabbit when inoculated intracerebrally; typical pruritus in rabbits and guinea pigs following subcutaneous inocu-

embryos and in guinea pigs by intracerebral inoculation also failed to show any relative increase in the tendency to destroy nervous tissue over the tendency to produce lesions on the membrane (Table II)

The fixed strains of pseudorabies are peculiar among chick embryo-cultivated viruses in that the hemorrhagic tendencies are produced by a virus recognizable by its intranuclear inclusions. It is therefore possible to tell whether the endothelium of the destroyed blood vessels is actually infected with virus. Brains infected with the fixed strains frequently showed such inclusions in the endothelium, while brains of embryos infected with recently isolated strains did not.

TABLE II
Effect of Intracerebral Passage of Iowa B Strain on Neurotropism

Virus	Dilution	Embryo, Average No of pocks	Result in guinea pigs
After 5 membrane passages	10^{-2}	3 2	2 of 2 killed
	10^{-4}	0 4	2 " 2 "
	10^{-5}	0	0 " 2 "
After 8 intracerebral rabbit passages	10^{-4}	24	3 " 3 "
	10^{-5}	4	2 " 3 "

TABLE III
Neutralization of Augesky Strain by Hyperimmune Serum

Dilution of virus	Virus + normal pig serum	Virus + hyperimmune serum
10^{-2}	Large central ulcers with scattered pocks	0,* 0, 0, 0
10^{-3}	6, 0, 34	0, 0, 0, 0

* Figures represent number of pocks on chorioallantoic membrane 2 days after inoculation

In order to identify the virus, neutralization tests were carried out several times, and it was possible to produce a consistent decrease in the pock count when the virus suspensions were combined with hyperimmune serum (Table III)

Embryos which failed to develop pocks also failed to show the usual hemorrhagic hydrocephalus

Chick embryos of varying ages differ in their reaction to a number of viruses (7, 11, 24). Infection by pseudorabies is no exception to this rule. This was shown by the simultaneous inoculation of 13, 15, and 18 day embryos on the chorioallantoic membrane, and the subcutaneous injection of virus into 2 day chicks (Table IV). A 1 per cent infected chick embryo suspension was used

Adult chickens are resistant to subcutaneous inoculation of the virus (15). This increase of resistance with age may be related to the increase in the temperature of chick embryos after the 14th day, for in two experiments, we have found that incubation at 40°C of 12 day embryos inoculated with pseudorabies produces fewer and more discrete lesions than at 37°. It is interesting to compare the results of pseudorabies in the 18 day embryo with those produced by rabies virus, as reported by Dawson (10). He finds that inoculation of chick adapted virus on the membrane fails to produce a lesion on the membrane, but will destroy the central nervous system.

Cellular reaction in the brain itself differs markedly with the different age groups, as shown in Figs 5 to 7. In 12 day embryos (16 day when fixed), the nerve tissue is extensively involved and hemorrhage is prominent. In 15 day embryos (20 day when fixed), although many nerve cells are destroyed, hemorrhage has ceased, a few leucocytes penetrate the brain tissue. In

TABLE IV
Effect of Age of Embryo on Type of Lesions

Age	No. inoculated	Reaction
<i>days</i>		
13	6	Large ulcers and confluent pocks
15	4	5 to 10 scattered pocks
18	7	No visible lesions on membrane subsequent encephalitis
2 day chick	4	2 developed encephalitis in 6 days

contrast to the embryo, there is no hemorrhage in the hatched chick and only slight destruction of nerve tissue. There is perivascular polymorphonuclear and mononuclear infiltration, with neuronophagia. For the first time the nerve tissue itself seems to react, for glial nodules are common.

DISCUSSION

Study of pseudorabies in the chick embryo has emphasized the neurotropic qualities of this virus. It is a unique infection, in that a reaction occurs both on the membrane and within the central nervous system. This is analogous to the natural disease in which a primary lesion develops at the point of inoculation and the virus subsequently infects the brain. Since there is no portal of entry to the embryo proper other than the blood stream, virus spreads to the embryo by this route and may be recovered from the embryo blood. This contrasts with the frequent neural spread of the natural disease, but recalls the humoral spread of certain strains (19) in the rabbit.

Some strains of influenza virus may simulate this disease pattern, but only after prolonged cultivation on the membrane (25, 26). Small pocklike lesions

develop on the membrane, and the influenza virus subsequently spreads to the embryo and produces a hemorrhagic destruction in the brain. This acquired neurotropism is confirmed by the ability of the virus to multiply in the brains of mice (26).

SUMMARY

The chick embryo responds to experimental infection with the virus of pseudorabies with a disease pattern simulating the natural infection. Virus lesions of the membrane are followed by infection of all tissues of the central nervous system.

Fixed strains produce a hemorrhagic destruction of the central nervous system of the embryo, which is referable to destruction of blood vessel endothelium. Field strains lack the hemorrhagic tendency, but infect the brain when inoculated on the membrane.

Neutralization of the virus by specific hyperimmune serum can be demonstrated by inoculation on the membrane.

The reaction of the embryo to the virus varies with the age of the embryo. This is reflected both in the membranal lesion and in the subsequent encephalitis.

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EXPLANATION OF PLATES

Sections stained with hematoxylin and eosin

These photographs were made by Mr Julian A Carlile

PLATE 15

FIG 1 Normal 16 day chick embryo on left. Embryo on right shows hemorrhagic bulging of cranium as a result of infection by Aujeszky strain of virus Slightly enlarged

FIG 2 Neurtus in embryo infected with Aujeszky strain Darkly staining cells are polymorphonuclears $\times 304$

FIG 3 Spinal cord of same chick showing destruction of nerve tissue $\times 111$

FIG 4 Meningitis in 16 day embryo following membranal inoculation $\times 100$

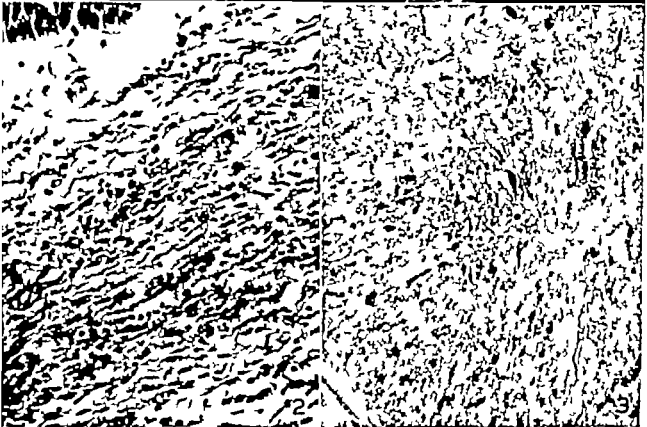


PLATE 16

FIG 5 Destruction and hemorrhage in 12 day embryo following membranal inoculation. If the infection had been allowed to develop, almost all nerve cells would have been destroyed. $\times 100$

FIG 6 Destruction of nerve cells in 15 day embryo following membranal inoculation. Note lack of hemorrhage and relative immunity of nerve cells at right. $\times 100$

FIG 7 Reaction in newly hatched chick following subcutaneous inoculation. Fewer cells are destroyed. $\times 100$



STUDIES IN THE PATHOGENESIS OF EXPERIMENTAL DYSENTERY INTOXICATION

By ABRAHAM PENNER, M.D., AND ALICE IDA BERNHEIM M.D

(From the Laboratories of The Mount Sinai Hospital, New York)

PLATES 17 AND 18

(Received for publication, March 10, 1942)

Previous to the bacteriologic era in the study of dysentery, this disease was looked upon as a systemic affection of which the colonic symptoms were considered a local manifestation. This was the view of Sydenham (1) as well as of Conradi (2) who actually conceived the colonic manifestations as being the result of the systemic disturbances. With the advent of the bacteriologic era the investigation of this disease was turned over to the laboratory and there followed a period of brilliant work in which the causative organisms were discovered. During this period however dysentery came to be viewed as a local disease, the result of the action of the causative organisms or their toxins upon the intestinal wall. The clinical phenomena of the disease were ignored except in so far as they were interpreted as the consequences of the colonic lesion. However, it soon became clear that this view point was too narrow. Thus, the pathologist realized that while the intestinal changes in epidemic dysentery represented a diphtheritic process, not every diphtheritic intestinal lesion was a case of dysentery. The clinician reentered the picture and pointed out that the clinical phenomena indicated the presence of a period of toxemia which preceded the onset of intestinal symptoms by several days and frequently persisted for some time thereafter.

The intestinal lesion in bacillary dysentery is a diphtheritic, ulcerative lesion at least in the advanced stage in which it is customary to observe it at autopsy. This characterization, however, is not in itself diagnostic of the disease. As we have shown elsewhere (3, 4), such lesions may occur as a result of the homeostatic responses called into play by various types of stimuli which may produce a shock like state. These anatomical changes, therefore, are not specific as to etiology.

In studying the various stages of evolution of these lesions, we were impressed by the observation that the earliest anatomical changes in the intestine were noted in the submucosa (3). We were able to trace the successive events leading to necrosis of the epithelial lining of the intestine and finally to pseudomembrane formation and ulceration. An attempt to stimulate the vasomotor responses by means of adrenalin injections (5) enabled us to reproduce the entire series of events leading to the production of the typical lesion. In these experiments we noted that the first anatomical changes occurred in the submucosa in the rabbit and guinea pig. In the former there was also a distinct predilection for the lesion to occur in the cecum. This preferential involvement of the cecum in the rabbit has been noted to occur following the injection of dysentery toxins (6-8). Even more striking, however is the fact that the initial changes observed in Shiga intoxication occur in the

submucosa (6-8) We felt that these phenomena might indicate a common pathogenetic mechanism

Biochemical studies of the effects of toxins upon the organism first began when Hibbard and Morrissey (9) noted glycosuria in patients with diphtheria Numerous investigators using a variety of procedures have adduced a great mass of evidence indicating that there is an increased tone in the sympathetic nervous system during bacterial intoxication (10-17) The use of several types of bacteria in these investigations indicates the lack of specificity in the physiological responses Thus, a diabetic glucose tolerance curve has been noted in diphtheria This can be prevented by the use of ergotamine tartrate (11) Others have been able to prevent the hyperglycemia by means of double splanchnotomy (12), or unilateral adrenalectomy and denervation of the opposite suprarenal gland (14) Finally, an increase in output of adrenalin from the suprarenals has been observed during toxemia resulting from either diphtheria toxin (16) or Shiga toxin (17)

We have been particularly interested in the effect upon the organism of the toxin of *Bacillus shigae* Despite the emphasis placed by the biochemist upon alterations in carbohydrate metabolism in diphtheritic intoxication, clinical diphtheria is much more characterized by vasomotor than by metabolic changes For this reason we decided to investigate some physical characteristics of the blood in acute Shiga intoxication We were led to do this because we have been able to find only one report (Yannet and Darrow) (18) of similar studies in diphtheritic intoxication Therefore, we have studied the effect in dogs and rabbits of the injection of the toxin of *B shigae* upon the hematocrit findings, specific gravity of the blood, hemoglobin, red blood cell count, white blood cell count, and differential cell count We have attempted to correlate the time of appearance of changes in these findings with the time of development of the anatomical lesions

Methods

The toxin was prepared from a smooth strain of *B shigae* according to the "slow method" of Olitsky and Kligler (19) No systematic effort was made to standardize this toxin Its potency, however, was such that 0.25 cc. per kg intravenously, killed a rabbit within 48 hours

Hematocrit readings were made by the method of Wintrobe (20) The specific gravity both of whole blood and of the plasma was determined by the falling drop method as devised by Barbour and Hamilton (21) The hematologic studies were performed with standard pipettes and chambers, the hemoglobin by the Sahli method, the smears stained with Jenner-Giemsa

Observations were made on ten dogs and four rabbits No anesthesia was used In addition, anatomical studies were made on 8 cats In the earlier experiments a control period of from 3 to 10 days was used, during which we studied the normal variations in the hematologic values These proved to be so small in comparison with the changes resulting from the injection of the toxin that this control period was subsequently omitted The various determinations were made in duplicate

On the day of experiment, following an 18 hour fast, a control series of observations were made. Immediately following this, the toxin was injected intravenously. The dogs received 0.5 cc. per kg., while the rabbits were given 0.25 cc. per kg., and the cats 1.0 cc. to 6.0 cc. per kg. At intervals of 15 to 30 minutes specimens of blood were withdrawn from the saphenous vein in the dog and the ear vein in the rabbit. An attempt was made to avoid the use of a tourniquet but after the first couple of specimens were obtained, venous filling was frequently so poor that a tourniquet was necessary. However, the specimens rarely took more than 1 minute to withdraw so that the factor of stasis is not important. As an anticoagulant we used an aqueous solution of 6 per cent ammonium oxalate and 4 per cent potassium oxalate. 0.2 cc. of this solution was used to 18 cc. of blood. The blood so obtained was employed in all our determinations. This anticoagulant solution caused no shrinkage or swelling of the red blood cells. The specific gravity of the plasma was determined on the supernatant fluid from the centrifuged hematocrit specimen. These determinations were made immediately upon withdrawal of the blood.

In addition, observations were made on the reactions of the animals to the injection of the toxin. In an attempt to correlate the physiologic changes with the time of appearance of the anatomical lesions, we sacrificed the animals at varying periods after the injection of the toxin. As a control study we injected a sterile broth culture medium which had been incubated and treated in the same fashion as our cultures of *B. shigae*. In addition we also used Shiga toxin inactivated by heat as suggested by Olitsky and Kligler (19). The dosage was five times that used for the toxin. We may add that such material caused no changes in blood composition and no anatomical changes were found in the intestinal tract upon sacrificing the animals. They were killed by the intravenous injection of air, chloroform, or 20 per cent formalin. This was done in order to eliminate in so far as possible the influence of the method of sacrificing the animal upon the anatomical changes. The organs were immediately removed and fixed in 20 per cent formalin.

In view of the demonstration by physiologic methods of a sympathomimetic reaction (10-17) resulting from the action of bacterial toxins we felt that a direct demonstration of its vasoconstrictor component would be desirable. We attempted to demonstrate this by means of the injection of India ink. We administered Shiga toxin to a group of five rabbits in a dose of 0.5 cc. to 1.0 cc. per kg. of body weight. After a time interval of from 3/4 hours to 4 hours we injected 10 cc. of India ink (Higgins American drawing ink, soluble, black) into the left ventricle, followed in 1 minute by an intravenous injection of 1 cc. of 40 per cent formalin. Control rabbits were similarly injected with India ink only and sacrificed in the same manner.

We found that this method while satisfactory for rabbits, was not suitable for the demonstration of vasomotor reactions in the dog's intestine due to the thickness of the latter which rendered transillumination impossible. For this reason we employed the following method. Three dogs were given Shiga toxin intravenously in a dose of 1 cc. per kg. body weight. They were sacrificed at intervals of from 7 to 11 minutes by the intravenous injection of 10 cc. of 40 per cent formalin. Paired controls were simultaneously sacrificed. The digestive tracts of the controls and of the dogs receiving the Shiga toxin were rapidly removed, washed thoroughly with

running water, and opened. The mucosa on the inner surface was carefully rinsed off and the bowel immersed in a saturated solution of benzidine (500 cc.), acidified with glacial acetic acid (10 cc.) and containing a small amount of hydrogen peroxide (20 cc.) The intestinal loops were then washed off in running water. The benzidine solution produced the usual deep blue color on contact with the red blood cells in the mucosal layer of the bowel wall.

In order to study the direct action of Shiga toxin upon the intestinal mucosa, we established typical Thiry Vella loops in three dogs, located in mid-jejunum. This segment of the intestine was chosen because it had practically never been involved in the dogs to which the toxin had been administered intravenously. After complete recovery, 35 to 50 cc. of the toxin were introduced into the loop at approximately 8 hour intervals, for a period of 24 hours. The animals were sacrificed as above, 8 to 12 hours after the administration of the last dose of toxin.

RESULTS

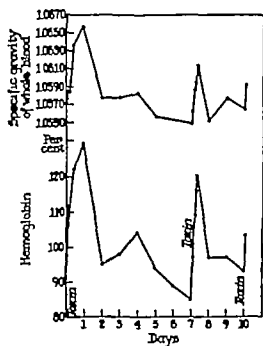
Course of the Intoxication—Our most detailed observations were made on the dogs. These animals were selected for their liveliness and frisky dispositions. For the first 15 or 20 minutes following an injection of toxin there was usually no evidence of any change in their behavior. Shortly after this, however, they developed periodically recurring episodes of tachypnea during which their tongues would protrude and their pupils dilate. This continued for perhaps 30 to 45 minutes during which the animals were content to lie quietly, except for the periods of tachypnea when they would rise to pace restlessly. At this time the dogs would begin to retch and salivate and not infrequently vomit small amounts of mucoid fluid. As this continued and increased in degree some of the animals showed some blood streaking in the vomitus. Shortly thereafter we not infrequently noted the passage of a normal formed bowel movement. This, however, was soon followed by the appearance of a marked diarrhea. At first the rectal discharges consisted of a mucoid fluid, which, however, was soon followed by blood streaking and eventually by a bloody stool. During this period the animals appeared weak. They could not be induced to take food or fluids. This state persisted for about 18 hours following which they gradually returned to their normal behavior. There was no significant rise in temperature during the 24 hours following the injection of the toxin. The pulse rate increased markedly during the earlier phases of the experiment and not infrequently reached levels of 150 to 160 per minute.

Hematologic Observations—As can be seen from Table I, and Text-fig 1 which are representative of our data, there was a rapid and parallel rise in the hemoglobin, red blood cell count, hematocrit reading, and specific gravity of the whole blood. These indices of blood concentration and diminution of blood volume began within 15 to 30 minutes following the injection of the toxin. They persisted for a period of 24 hours after the injection. They

could be elicited again at this time by reinjection of toxin but the response was not as marked as on the first injection. The specific gravity of the plasma showed no significant alterations throughout the experiments. This would

TABLE I
Dog 3-70

Date	Time	Hemo- globin	R.B.C.	W.B.C.	P.M.N.	Hema- tocrit	Specific gravity	Remarks
		<i>per cent</i>	<i>millions</i>	<i>thou sands</i>	<i>per cent</i>			
12/29/40	9 00 a.m.	92	6 27	12 4	78	44	1 0571	6 cc. Shiga toxin intravenously
	9 07							
	9 32	102	7 26	10 4	82	46	1 0577	Mucoid stool Weak
	9 50							
	10 00	107	6 93	3 8	52	49	1 0585	Bloody mucoid stool
	10 30	110	7 43	4 2	56	50	1 0585	
12/30/40	12 55 p.m.	122	7 39	8 2	88	57	1 0636	
	9 30 a.m.	129	8 64	49 8	94	57	1 0657	



TEXT FIG. 1

exclude vomiting and diarrhea as the cause of the blood concentration. These observations confirm those of Yannet and Darrow (18) in diphtheritic intoxication.

Anatomical Observations—Despite the early appearance of evidences of hemoconcentration and of diminution of blood volume no anatomical changes

appeared thickened. Hemorrhage by diapedesis then appeared in the mucosa and at this stage the epithelial cells began to undergo focal necrosis, followed by the formation of erosions.

In the experiments designed to test the direct action of the toxin, the mucosa of the Thiry-Vella loops in direct contact with the toxin showed no significant gross changes. The remainder of the gastrointestinal tract was carefully dissected and changes were noted in the duodenum, beginning at the pylorus and extending into the most proximal jejunum. These consisted of varying degrees of congestion which in its extreme form was hemorrhagic (Fig 7). Very much milder changes were noted in the preterminal ileum, extending to within 10 to 15 cm of the ileocecal valve. In general, the intensity of the changes was somewhat less than that observed following the intravenous injection of the toxin. In one of three dogs, the proximal portion of the Thiry-Vella loop showed a mild congestion. This loop was found at necropsy to have been made in the upper jejunum, which showed a similar mild congestion, extending to the entero-anastomosis which reestablished the continuity of the gut. Microscopically, we were able to discern the entire sequence of events typical of the histological changes resulting from the intravenous injection of Shiga toxin. However, in agreement with the gross observations, we did not achieve ulceration or pseudomembrane formation.

DISCUSSION

We have demonstrated a very rapid occurrence of hemoconcentration following the intravenous administration of Shiga toxin. As has been pointed out by Bogert, Underhill, and Mendel (24), as well as by Lamson and Roca (25), and by Darrow, Yannet, and Cary (26), one may use the hemoglobin concentration as an indicator of relative changes in blood volume under conditions such as obtained in our experiments. This being the case, the above data indicate a marked diminution in blood volume associated with an increase in blood concentration. This, taken in conjunction with an unaltered specific gravity of the plasma, indicates that the diminution in blood volume is due to a loss of plasma as such from the actively circulating blood. That the blood concentration is not due to vomiting or diarrhea is shown by the fact that it frequently occurs before these take place. In addition, the unaltered specific gravity of the plasma indicates that the fluid which has left the blood stream has a composition identical with that of plasma. We are as yet unable to state whether the plasma which is lost has left the actively circulating blood stream and is located in the tissues or is to be found in capillaries in which physical slowing of the current has resulted in an excessive amount of plasma in proportion to cells, as described by Cohnheim (27).

The relative changes in blood volume calculated on the basis of hemoglobin change would indicate in some of our experiments a diminution of blood

volume in excess of 30 per cent. Some idea as to the change in blood viscosity may be obtained from the fact that in dog 3-70 the hemoglobin rose to 129 per cent, associated with a rise in hematocrit to 57 per cent.

It is interesting to note that evidence of diminished blood volume which we observed occurs at a time when others have noted the presence of a hyperglycemia which has so frequently been found in states of intoxication (12, 14) As we have mentioned above, the preponderance of evidence indicates that the initial hyperglycemia at least is dependent for its appearance upon intact suprarenal glands under nervous control (12) The fact that denervation of the suprarenal gland prevents such a hyperglycemia is strong evidence in favor of this concept (12) This is also supported by the observation that ergotamine tartrate serves to prevent the hyperglycemia not only in the dog and rabbit but also in man (11)

Furthermore, as Hoshi (17) has shown by direct estimation of the adrenalin content of the suprarenal veins, injection of dysentery toxin leads to an increased secretion of adrenalin which begins about 30 minutes after injection, reaches its maximum in 2 to 4 hours, and then gradually decreases He also was able to show that the hyperglycemia which resulted, appeared simultaneously with the increase in adrenalin output and ran parallel to it The time of onset of the hyperglycemia as well as the increase in blood concentration coincides with that at which we noted evidence of vasoconstriction by the India ink and benzidine methods. The areas in which the vasoconstriction was noted in the rabbit and dog are identical with those in which gross and microscopic lesions appeared in experiments of longer duration These areas are the same as those in which we were able to produce intestinal lesions by the intraperitoneal injection of adrenalin In the cat both adrenalin and Shiga toxin caused a lesion with a predominant localization in the colon In the dog both resulted in a lesion located mainly in the duodenum and in the preterminal ileum. In the rabbit both substances acted to cause anatomical changes in the cecum This similarity in gross localization is even more striking when we compare the pathogenetic process as revealed microscopically In the cat and dog, both adrenalin and Shiga toxin produce a lesion which makes its appearance first in the villi It begins with evidences of ischemia followed by congestion which is in turn succeeded by a subepithelial edema, leading eventually to pseudomembrane formation In the rabbit, on the other hand, both adrenalin and Shiga toxin cause changes which first manifest themselves by focal ischemia followed by submucosal congestion of the cecum. This in turn is followed by focal submucosal edema, which is only later seen to involve the mucosa and lead to hemorrhage and ulceration

Because of the focal nature of the lesions and the difference in pathogenesis as observed in the dog and rabbit, we were led (5) in the case of the lesions

resulting from the injection of adrenalin to interpret the sequence of events in the light of the differences in vascular arrangement in the intestine between the dog and rabbit as noted by Spanner (28). We suggested that a vasoconstriction involving the other terminal arterioles than the ones participating in the arteriovenous anastomosis, would serve to cause a deprivation of blood supply to the submucosa in the case of the rabbit. A similar sequence of events in the cat and dog leads to an ischemia involving the mucosa. The identity in the sequence of events here described resulting from the injection of Shiga toxin with those caused by adrenalin compels us to view the lesions caused by the administration of Shiga toxin as being vascular in origin.

The experiments in which the intestinal mucosa was brought into direct contact with the toxin indicates that the latter does not act directly upon the mucosa. This is supported by the observations of Doerr (6) and Flexner and Sweet (7) who described the earliest changes resulting from the intravenous injection of Shiga toxin as appearing in the submucosa of the rabbit. This is also what happens in bacillary dysentery in man as shown by Heubner (29), Lohlein (30), and Castellanos (31). Furthermore, in the cat and dog in which the mucosal layer is primarily affected, the earliest changes are seen not in the epithelial cells but in the stroma beneath them, as we have described above. In addition, Barg (32) using the method of tissue culture reported that the concentration of Shiga toxin necessary to inhibit growth and kill explants of intestinal mucosa was extremely high. He noted that even this reaction was not specific in that it occurred after heating and destroying the potency of the toxin.

In our original studies of the diphtheritic ulcerative lesions observed at necropsy in a variety of clinical conditions (3, 4), we were impressed by the fact that these lesions occurred in patients who had succumbed after a period of shock. The anatomical features of these lesions compelled us to consider them to be vascular in origin. The absence of any organic vascular lesion led us to the view that an intense vasospasm resulted in an ischemic necrosis of the tissues. The cause of such a vasospasm was obviously not local, since we were able to observe lesions throughout the entire digestive tract as well as in the liver and kidneys. The concept introduced by Cannon of a compensatory vasoconstriction serving to adjust the capacity of the vascular bed to the diminished blood volume caused by a shocking stimulus (33), offered an explanation for the anatomical lesions. As Cannon has shown, this compensatory vasoconstriction is dependent upon intact suprarenal glands and sympathetic nervous system. Stimulation of the sympathetic vasomotor reactions by means of the intraperitoneal injection of adrenalin enabled us to reproduce the entire sequence of events leading to intestinal lesions such as we had observed in our clinical material. We have demonstrated above that Shiga toxin causes hemoconcentration and a diminution in blood volume.

These phenomena occur simultaneously with anatomic evidences of focal vasoconstriction in areas which subsequently develop typical diphtheritic ulcerative changes. This sequence of events is typical of the vasomotor homeostasis described by Cannon. The participation of the sympathico-suprarenal system in this process is shown by the fact that the toxin also causes a hyperglycemia which runs parallel with the discharge of adrenalin from the suprarenal glands (17). We therefore interpret the lesions caused by the toxin of *B. shigae* as being the result of a homeostatic vasoconstriction, mediated through the sympathico-suprarenal system.

CONCLUSIONS

The intravenous injection of Shiga toxin into dogs causes a rise in hemoglobin, red blood cell count, hematocrit reading, and specific gravity of the whole blood. There is thus a decrease in circulating blood volume. The specific gravity of the blood plasma does not change. These findings indicate that the toxin of *B. shigae* produces a shock like circulatory state. As a result there occurs a compensatory vasoconstriction in the duodenum of the dog and in the cecum of the rabbit. It has been shown that the toxin of *B. shigae* has no direct effect upon the intestinal mucosa when brought into contact therewith, but that its absorption through the mucosa leads to the appearance of a lesion in the duodenum of the dog. Therefore we interpret the pathological alterations in the intestinal tract, following the injection of Shiga toxin, as the anatomic end result of a pronounced and prolonged homeostatic vasoconstriction.

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EXPLANATION OF PLATES

PLATE 17

FIG 1 Cecums of rabbits which had received 10 cc. of India ink into the left ventricle and were sacrificed 1 minute later *a*, cecum of the control rabbit (No 7-91), *b*, cecum of the rabbit (No 8-14) which received Shiga toxin, 1 cc per kg 1 hour before the India ink injection 0.8 natural size

FIG 2 Cecum of rabbit (No 8-16) which received 0.5 cc per kg Shiga toxin intravenously followed in 2 hours by 10 cc of India ink into the left ventricle and sacrificed 1 minute later Note the focal areas in the folds, devoid of ink, some of which have fused into larger areas $\times 12$

FIG 3 Dog stomachs and duodenums stained with benzidine *a*, from a normal control dog (No 1-16), *b*, from a dog (No 1-13) which received Shiga toxin, 1 cc per kg intravenously, 7 minutes before being sacrificed $\times 0.4$

FIG 4 Dog (No 4-78) Gross appearance of a duodenal lesion resulting from the intravenous injection of Shiga toxin Note its sharp upper limit at the pylorus Killed 6 hours after the injection

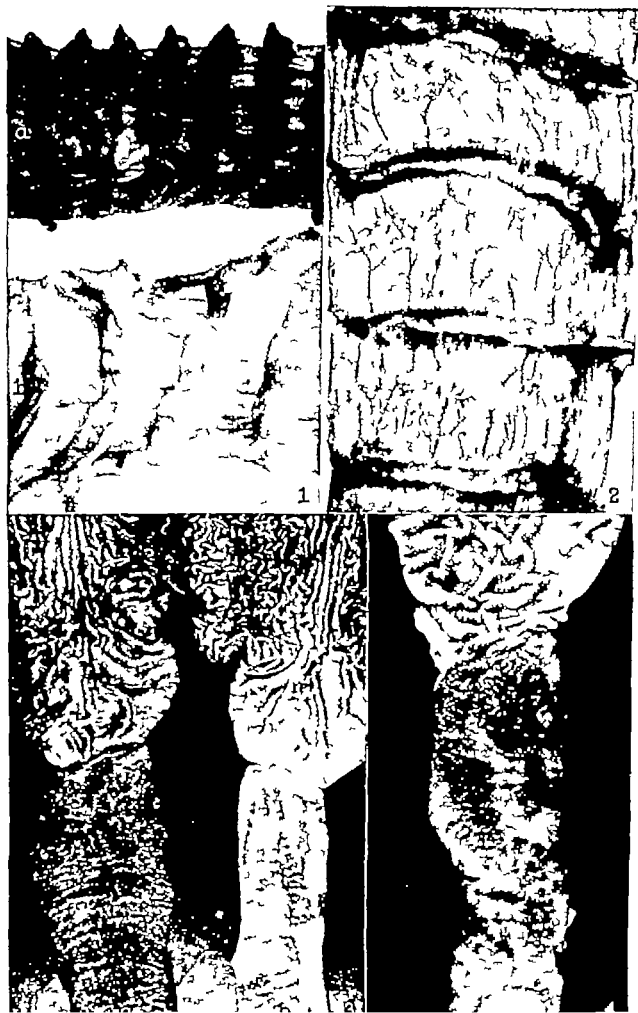


PLATE 18

FIG 5 Dog (No 4-78) High power view of duodenal villi, showing a separation of the epithelium from the underlying stroma, caused by an accumulation of plasma and blood cells Hematoxylin and eosin $\times 212$

FIG 6 Dog (No 4-78) Duodenum showing membranous enteritis with normal villi adjacent to a focally involved villus which is enmeshed in the membrane Hematoxylin and eosin $\times 72$

FIG 7 Dog (No 4-66) Stomach and duodenum, and Thyry Vella loop The latter, seen below, is unaffected by the Shiga toxin which had been introduced into it, whereas the former presents a lesion indistinguishable from that caused by the intravenous administration of the toxin



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HEMOGLOBIN PRODUCTION FACTORS IN THE HUMAN LIVER ANEMIAS, HYPOPROTEINEMIA, CIRRHOSIS, PIGMENT ABNORMALITIES, AND PREGNANCY

BY G. H. WHIPPLE, M.D., AND F. S. ROBSCHT ROBBINS, PH.D.

(From the Department of Pathology The University of Rochester School of Medicine and
Dentistry Rochester, New York)

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The most significant observations in the tables below relate to *hypoproteinemia*, pregnancy, and the postpartum state. With the reduction of protein stores which must accompany hypoproteinemia, we observe in practically all cases that the biological assay of these livers shows only $\frac{1}{3}$ to $\frac{1}{2}$ of the normal store of hemoglobin producing materials.

It is generally believed that the liver stores many factors essential for hemoglobin building—for example it holds the major stores of iron and the essential factor missing in pernicious anemia. We believe that the liver stores some of all the factors essential for hemoglobin building and it is not difficult to show that the building of hemoglobin can be limited by reducing protein stores and intake in experimental anemia (2, 6). This might indicate that the liver is concerned with the production and/or storage of globin or its precursors.

The pigment radicle of hemoglobin—the pyrrole aggregate—we know is discarded by the liver (bile pigment) and possibly the pigment radicle is made in the same organ. It does not seem possible to exhaust the capacity of the body to produce this pigment radicle—perhaps the mechanism being a ring closure of straight chain amino acids.

Some years ago we reported analyses of human livers (12). The iron content was determined and a biological assay of the hemoglobin-producing factors in human tissue was made by the utilization of carefully standardized anemic dogs. Our control baseline for the normal animal liver is that of the pig and we designate that as 100 per cent. Compared with this 100 per cent normal control we find that the normal human liver contains greater amounts of hemoglobin-producing factors—a ratio of 120 to 160 per cent to the control. There are considerable individual differences which we are inclined to relate to dietary habits, iron stores, and other unknown factors.

Subsequently we made biological assays upon the livers from common domestic animals (8). Compared with the hemoglobin production resulting from pig liver feeding as 100 per cent, we found beef liver rated at 70 per cent, rabbit liver 80 per cent, dog liver 100 per cent, and horse liver 130 per cent. Fish livers in contrast were relatively inert—perhaps 10 per cent being the upper limit for the various salt water fish tested (7, 10).

In our study of the biological assays of human livers (12) we observed that the usual acute and chronic infections showed about the same ratio as did the normal human group—average figures of 120 to 150 per cent as compared with 100 per cent for the normal control pig liver. *Cirrhosis* with no sign of liver insufficiency presented high normal values—160 per cent, but when there was evidence of a true hepatic insufficiency, the liver assay averaged only 50 per cent or $\frac{1}{3}$ normal. The iron content was the same in both types of cirrhosis.

Pernicious and *aplastic anemias* showed large stores of iron and a high assay for hemoglobin production 220 to 200 per cent. The usual secondary anemias showed the biological assay to fall within normal range—130 per cent with low iron values.

Other experiments with *anemic horse liver* (14) show that it is not easy to exhaust the hemoglobin production factors from the liver by means of blood loss. Iron stores can be reduced very readily by blood removal. These and other experiments suggest that the important reserve stores for hemoglobin building are in part *protein* and are guarded jealously by the body even in the face of severe bleeding. We must diminish the protein intake to reduce this protein fraction of the hemoglobin production store (6).

Obviously a comprehensive understanding of hemoglobin fabrication within the body in health and disease calls for an analysis of the stores of hemoglobin-producing materials in animal and human livers. This knowledge may make for a better therapy in human disease.

Methods

Methods used have been described previously (15). The standard dogs are kept anemic at a level of 6 to 7 gm hemoglobin per 100 cc blood by bleeding and the hemoglobin removed is credited to that test period. During control periods the dogs are fed a complete diet—a salmon bread mixture (15) which is poor in iron but adequate for health and general nutritional maintenance indefinitely. The dogs are standardized by the feeding of pig liver at various times. Similar control tests are made by feeding iron during other test periods as described elsewhere (11). The average hemoglobin production due to pig liver feeding (300 gm per day for 7 days) is 35 to 45 gm. When the human liver is tested, proper adjustment is made for the amount of liver used and this is then compared with the pig liver baseline as 100 per cent. The figures for this *ratio* are given in the last column in the tables.

In all these test experiments data on red cell counts, hemoglobin levels, blood volume, and animal condition are available in the histories of the various dogs but are not given in this report. These dogs were in a normal clinical state at all times.

Iron analyses were made by the method described by Kennedy (3). These figures are somewhat higher than would be found in perfused livers which give values related to the parenchyma iron only. Significant clinical diagnoses and a brief description of the liver are given under the autopsy number.

EXPERIMENTAL OBSERVATIONS

The experiments given in the tables below extend and confirm those previously reported (12). These data make for a clearer vision relative to hemoglobin production in various diseases.

TABLE 1
Pernicious Anemia
Hemoglobin Production Factors Much Increased

No.	Diagnosis	Liver intake per day	Liver iron content	Hemoglobin production from 7 day feeding of liver	Ratio Human liver to control liver Hb production
		gm	mg per cent	gm	per cent
2100	Pernicious anemia typical—no therapy	180	159	63	256
4609	Pernicious anemia, typical—no therapy	260	133	57	194
2262	Pernicious anemia, typical—no therapy	195	34	72	264
4394	Pernicious anemia—no therapy	270	164	73	253
6317	Pernicious anemia—slight therapy	166	95	47	257
3547	Pernicious anemia—slight therapy	175	21	56	192
5255	Pernicious anemia—slight therapy	132	23	53	300
6919	Pernicious anemia—slight therapy	154	57	48	254
4540	Pernicious anemia—good therapy	300	15	67	172
3447	Pernicious anemia—atypical	160	14	41	175
Average		199	72	58	232 (243)

Table 1 *Diagnosis and histological description of liver*

2100 Pernicious anemia typical—no liver therapy—terminal infection—hemoglobin 5 gm—73 years.

Liver—autopsy weight 1500 gm.

Histological specimen—liver cells, in general, normal. They contain some fat droplets and many fine yellow pigment granules which give a positive stain for iron. Kupfer cells contain some of the same pigment. There are mononuclear cells in the periportal stroma—old cholangitis?

4609 Pernicious anemia, typical,—no liver therapy, hemoglobin 3.2 gm.—76 years.

Liver—autopsy weight 1870 gm

Histological specimen—liver cells in center of lobules show fatty degeneration and considerable hyaline necrosis. Liver cells elsewhere are rich in fine granular pigment which gives a strong stain for iron. Kupfer cells show pigment.

2262. Pernicious anemia, typical,—no therapy—bronchopneumonia—hemoglobin 3.9 gm—63 years.

Liver—autopsy weight 1610 gm.

Histological specimen—Liver cells in general normal. Few granules of yellow pigment in hepatic epithelium.

4394 Pernicious anemia—transfusion reaction—no therapy—hemoglobin 3.4 gm — 64 years

Liver—autopsy weight 2000 gm

Histological specimen—liver cells excluding the large central necroses are normal. Pigment is abundant in liver cells and it gives a deep iron stain. Kupffer cells small and contain little pigment. Bone marrow is not hyperplastic but shows atrophy in ribs and vertebrae. The marrow which remains is compatible with the diagnosis of pernicious anemia.

6317 Pernicious anemia, typical,—occasional short liver therapy during 5 years—hemoglobin 4 gm —63 years

Liver—autopsy weight 1410 gm

Histological specimen—liver cells in center of lobules show atrophy, some fatty degeneration and hypochrome pigment. Liver cells in margins of lobules show abundant iron staining pigment. Kupffer cells are filled with iron staining pigment. Bone marrow typical. Kidney epithelium in convoluted tubules contains iron staining pigment.

3547 Pernicious anemia—little therapy and no response—coronary occlusion—hemoglobin 5.7 gm —59 years

Liver—autopsy weight 1550 gm

Histological specimen—liver cells normal. They contain many fine pigment granules some of which give a stain for iron. Kupffer cells contain some pigment. Scattered central hyaline necroses.

5255 Pernicious anemia—occasional liver therapy 2 years previously with relapse—moderate response to therapy in last week—arteriosclerosis—final cardiac death—hemoglobin 4.8 gm —65 years

Liver—autopsy weight 1030 gm

Histological specimen—liver cells show atrophy and some fat droplets. The central liver cells show yellow pigment but the iron stain is faint. Kupffer cells inconspicuous and non-pigmented.

6919 Pernicious anemia—treatment 2 weeks "reticulogen"—pyelonephritis—bronchopneumonia—hemoglobin 7.3 gm —red blood cells 1,700,000 to 3,000,000 under therapy—70 years

Liver—autopsy weight 1430 gm

Histological specimen—liver cells show fatty degeneration and some atrophy. Abundant iron staining pigment in liver cells and Kupffer cells. Kidney cells in convoluted tubules contain abundant iron staining pigment. Atrophic gastritis.

4540 Pernicious anemia—erysipelas—liver therapy for years—cirrhosis and cholelithiasis—hemoglobin 15 gm —73 years

Liver—autopsy weight 3080 gm

Histological specimen—liver cells in margin of lobules show a good deal of fat but little if any pigment. There is much periportal stroma which is rich in mononuclear cells—the cirrhosis is probably related to a cholangitis. Liver lobules are distorted but liver cells are in good condition. The pernicious anemia was under control.

3447 Anemia (pernicious?)—some reticulocyte response to liver therapy—coronary occlusion with infarct—hemoglobin 8 gm —74 years

Liver—autopsy weight 1200 gm

Histological specimen—liver cells show atrophy. There are scattered small liver necroses. A few granules of iron staining pigment are found in liver cells and Kupffer cells. Marrow shows no typical hyperplasia. If this is pernicious anemia it must be a very early stage. The picture is not that of the ordinary secondary anemia.

Table 1 presents 10 cases of pernicious anemia, 8 of which are typical and had received no therapy or inadequate therapy. If we exclude the last 2 cases in Table 1, we obtain an average ratio of 243 per cent, which means that the pernicious anemia liver contains unusually large stores of hemoglobin-building factors. The missing substance X present in liver extracts, liver, stomach, and kidney tissue, is lacking and these unused substances pile up in reserve. When liver therapy is effective these liver stores decrease to normal and ratios close to the normal are observed (case 4540, Table 1 above) as new red cells are formed in great numbers.

The last 2 cases in Table 1 show high normal ratios for the hemoglobin producing factors. In 4540 the therapy had been adequate and there was no anemia at time of death. In case 3447 the anemia was not severe and the findings suggest an early stage of pernicious anemia.

Table 1 is very like the similar report on pernicious anemia ((13) Table 31). Average ratios for hemoglobin-producing factors in the liver previously reported were 218 per cent.

TABLE 2
Anemia—Aplastic and Secondary

No	Diagnosis	Liver Intake per day	Liver Iron content	Hemoglobin production from 7 day feeding of liver	Ratio Human liver to control liver Hb production
		gm.	mg per cent	gm	per cent
4395	Aplastic anemia	205	244	65	280
6690	Aplastic anemia—benzol?	200	89	38	154
3958	Secondary anemia (macrocytic)	215	16	38	161
2817	Secondary anemia—nephritis	165	28	52	207
2433	Secondary anemia—cancer of cervix	125	4	21	125
4948	Secondary anemia—myocarditis	150	4	21	89
5407	Secondary anemia—therapy	180	7	15	60

Table 2 *Diagnosis and histological description of liver*

4395 Aplastic anemia—transfusions—hemoglobin 2.5 gm—white blood cells 500—no pigmentation—53 years.

Liver—autopsy weight 1570 gm.

Histological specimen—liver cells in general are normal. There are some fat vacuoles and scattered central hyaline necroses. Fine pigment granules which give an iron stain are abundant in liver and Kupffer cells.

6690 Aplastic anemia (benzol?)—bleeding—albumin 3.1 gm—globulin 2.4 gm—fibrinogen 533 mg—duration 2 months—hemoglobin 2 gm—73 years

Liver—autopsy weight 1570 gm

Histological specimen—there are small scattered central hyaline necroses. Pigment is scant in liver cells but obvious in Kupffer cells. Marrow shows few cells and those are immature.

3958 Anemia (macrocytic)—senility—cachexia—bronchopneumonia—hemoglobin 9.5 gm—84 years

Liver—autopsy weight 1500 gm

Histological specimen—there are mononuclear cells in the periportal stroma. Liver cells show lipochrome pigment but no iron staining pigment. Bone marrow does not suggest primary anemia.

2817 Anemia—chronic nephritis—death uremia—hemoglobin 5.2 gm—27 years

Liver—autopsy weight 1400 gm

Histological specimen—liver cells show some small fat droplets and lipochrome pigment but no iron staining pigment. Marrow shows no hyperplasia. Evidently in this type of anemia the iron and other hemoglobin building factors are not depleted from the liver—perhaps the mechanism of hemoglobin production is disturbed by the nephritis.

2433 Anemia secondary to cancer of cervix—red blood cells 1,000,000—hemoglobin 2 gm—38 years.

Liver—autopsy weight 1250 gm

Histological specimen—liver shows fatty degeneration of cells in the centers of lobules. Other liver cells show cloudy swelling. Scanty lipochrome pigment.

4948 Anemia, secondary—myocarditis—old gastroenterostomy—hemoglobin 4 to 6 gm—72 years

Liver—autopsy weight 1800 gm

Histological specimen—liver is negative except for some lipochrome pigment in the centers of the lobules. Bone marrow shows little if any hyperplasia. Iron stores obviously have been depleted.

5407 Anemia—gastric resection, subtotal, 7 years previously—hematuria—recovery from anemia with liver and iron therapy—death from meningitis—last hemoglobin 13 gm—63 years

Liver—autopsy weight 1540 gm

Histological specimen—liver cells essentially normal and contain a few grains of lipochrome pigment. Marrow is normal. The reserve of iron and other hemoglobin building materials in the liver has been exhausted.

Table 2 presents various types of anemia and the contrasts are striking not only in the iron stores but in other hemoglobin-producing factors. In the usual type of secondary anemia due to blood loss we expect to find very low iron stores and a low normal figure for hemoglobin production (135 per cent in a previous publication—Table 33 (13)). The last 3 cases in Table 2 illustrate well the low iron stores and also the decreased stores of hemoglobin-producing factors.

In contrast to these stands *aplastic anemia* (4395—Table 2), a case with very

large stores of iron and hemoglobin producing factors. The other aplastic anemia (6690) possibly due to benzol shows quite large iron stores but only average stores of hemoglobin producing factors. This unexpected low value may be explained in part by a definite hypoproteinemia which tends to reduce hemoglobin producing materials in contrast to aplastic anemia which tends to increase these stores. In part these large stores of iron and hemoglobin producing factors may be related to transfusions. The liver probably stores these essential hemoglobin producing factors because the bone marrow is aplastic and unable to use the stored material in spite of the demand due to the anemia.

Two cases (3958 and 2817 in Table 2) are not easy to understand. In spite of severe anemia of considerable duration, the liver stores of iron and hemoglobin-producing factors are large. In advanced nephritis in dogs there may be a lack of ability to make new hemoglobin under controlled experimental conditions (16). The supplies are adequate but the mechanism for hemoglobin production is disturbed just as may happen due to an infection (9). Perhaps this explains the observed stores (autopsy 2817—Table 2) and their non-utilization by a bone marrow which appears normal or hyperplastic. The other case of macrocytic anemia (3958) was observed in a male of 84 and here cachexia may have been a factor.

TABLE 3
Hypoproteinemia—Cirrhosis
Hemoglobin Producing Factors Greatly Decreased

No	Diagnosis	Liver Intake per day	Liver iron content	Hemoglobin production from 7 day feeding of liver	Ratio Human liver to control liver Hb production
		gm.	mg. per cent	gm.	per cent
6630	Carcinoma of stomach	200	4	4	17
6406	Gastric ulcer	160	5	12	61
6543	Lymphoma	350	10	29	52
3572	Tuberculosis	230	3	33	94
6892	Hepatitis—icterus	200	13	10	46
2237	Hepatic insufficiency	230	3	19	64
2824	Cirrhosis	245	2	37	90
5436	Pyelonephritis, ? pellagra	134	13	43	240
Average		219	7	23	83 (61)

Table 3 *Diagnosis and histological description of liver*

6630 Cancer of stomach—hypoproteinemia—peritonitis—considerable weight loss in 6 months—albumin 3.4 gm. globulin 2.5 gm.—hemoglobin 12.8 gm.—67 years.
Liver—autopsy weight 1400 gm.

Histological specimen—liver cells show atrophy and scattered fat droplets
Lipochrome present in moderate amount

6406 Gastric ulcer—anemia—hypoproteinemia—pyloric stenosis—phlegmonous gastritis—bronchopneumonia—pulmonary infarcts—bleeding ulcer for 4 years—total plasma proteins low = 3.8 to 6.0 gm per cent—hemoglobin 9 gm—35 years

Liver—autopsy weight 1250 gm

Histological specimen—liver cells show hyaline necrosis in the centers of all lobules. The midzone shows fatty degeneration. Lipochrome pigment easily seen.

6543 Lymphoma—hypoproteinemia—infiltration of ileum—bronchopneumonia with abscesses—total plasma proteins 6.1 gm to 4.3 gm per cent.—hemoglobin 12.8 to 8.5 gm—53 years

Liver—autopsy weight 2950 gm

Histological specimen—liver cells show fatty degeneration, especially in the periportal areas. There is some cell atrophy in the centers of the lobules.

3572 Tuberculosis, disseminated—anemia, macrocytic—hypoproteinemia—ascites—albumin 2.2 gm, globulin 2.0 gm—hemoglobin 10 gm—61 years

Liver—autopsy weight 1700 gm

Histological specimen—liver cells show advanced fatty degeneration. No tubercles. Marrow shows some hyperplasia but the cell elements are normal.

6892 Cirrhosis—hepatitis—liver insufficiency—coma—icterus, severe—hemoglobin 13 gm—39 years

Fibrinogen 360 mg per cent—albumin 1.6 gm per cent—globulin 2.3 gm per cent—N P N 45 to 130 mg per cent

Liver—autopsy weight 1800 gm

Histological specimen—typical portal type of cirrhosis with much scar tissue and new bile ducts. Little fatty degeneration but *considerable* areas of necrosis. Many polymorphonuclear leucocytes are observed in lobules and in cellular portal tissue. Larger bile passages are clear. Bile canaliculi are distended with brown colloid casts. Serious parenchyma injury.

2237 Cirrhosis—hemorrhages—anemia—hepatic insufficiency—alcoholism and syphilis—no blood clots—bleeding into tissues—icterus—hemoglobin 10.6 gm—44 years

Liver—autopsy weight 1690 gm

Histological specimen—typical portal type of cirrhosis with much scar tissue and new bile ducts. There is much fatty degeneration. Practically no pigment seen. Marrow shows some hyperplasia.

2824 Cirrhosis—icterus—bronchopneumonia—alcoholism—hemoglobin 10.2 gm. Albumin 2.1 gm, globulin 4.0 gm—fibrinogen 335 mg—61 years

Liver—autopsy weight 1930 gm

Histological specimen—liver cells show advanced fatty degeneration. Lipochrome pigment is abundant. Bile canaliculi are distended with yellow brown casts. The lobulation is irregular due to dense bands of scar tissue in which are seen many mononuclears and immature bile ducts. The liver is poor in iron and other hemoglobin building material.

5436 Hypoproteinemia (albumin 2.1 gm and globulin 2.6 gm)—hyperchromic anemia—pyelonephritis—pellagra?—restricted, inadequate diet months before death—hemoglobin 5.5 gm—52 years

Liver—autopsy weight 1740 gm.

Histological specimen—liver cells show much fatty degeneration especially in the centers of lobules—pigment very inconspicuous. There are a few small focal necroses. Kupffer cells show an occasional grain of pigment.

Table 3 is probably the most significant in this paper. With *hypoproteinemia* in all cases but one there is a sharp drop in the content of hemoglobin-producing factors. In fact if we exclude case 5436 from the group we find an average ratio of 61 per cent or about 40 per cent of the normal human liver content of hemoglobin production factors. Anemia is not prominent (excluding 5436) and the iron stores are depleted. The normal iron values for this type of human liver material is 12 mg per cent. We were surprised to note this uniformly low value for the hemoglobin producing factors and one can scarcely escape the conclusion that protein factors (perhaps precursors of *globin*) are depleted by the hypoproteinemia whether associated with hepatitis or not.

Case 5436 Table 3, does show a surplus of hemoglobin building stores and a normal store of iron in spite of an anemia (hemoglobin 5.5 gm.) plus a severe hypoproteinemia (4.7 gm per cent) preceded by a long period of inadequate diet (pellagra?) and pyelonephritis. We have no adequate explanation but note a pyelonephritis which like the nephritis in Table 2 may prevent the utilization of the hemoglobin-producing stores.

TABLE 4
Pigment Metabolism Abnormal
Hemolytic Icterus Hemochromatosis Erythroblastic Anemia

No.	Diagnosis	Liver intake per day	Liver iron content	Hemoglobin production from 7 day feeding of liver	Ratio Human liver to control liver Hb production
		gm.	mg per cent	gm	per cent
5340	Hemolytic icterus	250	80	50	177
3891	Hemolytic icterus	240	37	55	177
4226	Hemochromatosis	200	644	35	98
5812	Mediterranean anemia	240	418	50	155
2414	Mediterranean anemia	115	292	64	398

Table 4. *Diagnosis and histological description of liver*

5340 Hemolytic icterus—splenectomy—thrombosis femoral veins—pulmonary embolism—icterus—pigmentation of liver and kidneys—32 years.

Liver—autopsy weight 1800 gm

Histological specimen—liver shows recent hyaline central necrosis and many areas which show active repair of this injury. Pigment is abundant in the liver cells, phagocytes and Kupffer cells, also in renal tubular epithelium. Marrow shows very active hyperplasia.

Polycythemia (Table 5) shows a *low biological assay* and a subnormal iron store. These figures are significant and suggest that the iron and other hemoglobin-producing factors are turned over very rapidly to form new red cells to maintain the high red cell counts so characteristic of this disease. An *over-absorption* of red cell and hemoglobin-producing factors would not seem to be responsible for this disease picture. This case had been under observation and treatment with hydrazine in the Strong Memorial Hospital for 14 years. The presence of *iron* in the convoluted tubular epithelium of the *kidney* is related to the hydrazine therapy and emphasizes the fact observed in dogs (1) and human beings that the *kidney* is an organ concerned at times with *iron conservation*. Compare also the iron-containing pigment of the renal epithelium in hemolytic icterus cases—Table 4.

Leukemia (Table 5) presents an interesting picture. We reported a similar biological assay on 14 cases ((13) Table 34). In that report the iron content was about normal and the biological assay showed a ratio of 120 per cent or a low normal value. There are several factors which may influence the hemoglobin-producing stores in the liver in this disease. There may be bleeding which would deplete these liver stores but usually the iron stores are normal or above (Table 5) indicating that the drain due to bleeding is not serious. The marrow may be so choked with white cells that it can turn out too few red cells and anemia results. Meanwhile the stores of iron and hemoglobin-producing factors may heap up in the liver (case 4283—Table 5). Infiltration of the liver with white cells and associated liver degeneration may militate against the storage of protein hemoglobin-producing factors. The iron stores in the cases of leukemia (Table 5) run from 3 to 20 times normal.

TABLE 6
Eclampsia—Lactation—Controls

No.	Diagnosis	Liver intake per day	Liver iron content	Hemoglobin production from 7 day feeding of liver	Ratio Human liver to control liver Hb production
		gm	mg per cent	gm	per cent
4494	Eclampsia—7 mos	225	5	29	72
3694	Eclampsia—8½ mos	143	9	14	58
6369	Lactation—4 wks	223	4	16	60
4297	Thyroid storm	165	13	25	112
4148	Pneumonia—senile	128	8	30	163
6728	Hydrocephalus—youth	230	5	29	109

Table 6. *Diagnosis and histological description of liver*

4494 Eclampsia—7 months pregnancy—hypoproteinemia (albumin 14 and globulin 3.7 gm per cent)—39 years

Liver—autopsy weight 1810 gm

Histological specimen—there are numerous typical hyaline liver necroses with hemorrhage in the periphery of many lobules. The remaining liver cells show cloudy swelling and a few small fat droplets.

3694 Eclampsia—8½ months pregnancy—convulsions—coma—blood pressure 190—albumin and globulin 6 gm.—hemoglobin 11.7 gm.—25 years.

Liver—autopsy weight 1370 gm.

Histological specimen—liver is normal in gross as well as under the microscope. Many sections examined but none shows any periportal or other necrosis. Kidneys show lesions typical of eclampsia. Possibly the normal plasma proteins gave some protection against liver injury.

6369 Postpartum (4 weeks) lactation—pulmonary embolism—hemoglobin 10.2 gm.—23 years.

Liver—autopsy weight 2050 gm

Histological specimen—liver is normal. Cells contain a few small fat globules and glycogen granules.

4297 Thyrotoxicosis—postoperative bronchopneumonia—hypertension 200/80—red blood cells 2 800 000—hemoglobin 7 gm.—67 years.

Liver—autopsy weight 1170 gm.

Histological specimen—liver cells show atrophy a little fat infiltration and some lipochrome pigment normal for an elderly female.

4148 Pneumonia—senile—hemoglobin 12.6 gm.—80 years.

Liver—autopsy weight 1800 gm

Histological specimen—liver cells show cloudy swelling and small amount of lipochrome pigment.

6728 Hydrocephalus—meningitis—normal organs except brain—well developed—hemoglobin 16 gm.—16 years.

Liver—autopsy weight 1790 gm

Histological specimen—liver cells quite normal.

Table 6 presents 3 important cases related to *pregnancy*. The evidence is clear from these and other cases reported previously ((13) Table 35), that the iron and protein stores are very low in late pregnancy. Case 4494 eclampsia shows hypoproteinemia—compare Table 3. Demands coming from the fetus are probably largely responsible but the needs for protein due to *lactation* are real and deplete protein stores in the liver which otherwise might go to form plasma protein or hemoglobin.

These observed facts should direct the attention of the clinician to adequate intake of iron and proteins for the woman in late pregnancy and during *lactation*. Whether these depleted protein reserves are in any way related to the state of eclampsia is not known but protein depletion is known to favor liver injury (5) and impair the defense against infection (4).

The last three cases in Table 6 show control values which are in line with many others reported earlier (12). Thyrotoxicosis does not cause any change

in the iron content or the biological assay of the liver. Pneumonia likewise presents figures within the normal range. The last case (hydrocephalus) was a healthy, well developed male of 16 years—death due to meningitis. The figures for liver iron are low but the circulating hemoglobin was normal. Biological assay of the liver shows hemoglobin-producing factors to be below normal possibly due to some diet limitations.

DISCUSSION

The anemia of leukemia has received a good deal of attention, is a well recognized fact, and might be explained in various ways. Loss of blood is often a diagnostic factor in leukemia but the observations in Table 5 indicate that in many cases it is not responsible for the anemia. There are large iron stores and normal or above normal reserves of hemoglobin-producing factors in the liver. *White cell infiltration* of the marrow and liver is probably responsible for the values recorded in Table 5. When the marrow is stuffed with white cells the *red cell* elements can not function properly and there is a tendency toward anemia with some overaccumulation of hemoglobin-producing factors in the liver. There is no evidence of lack of absorption of iron or protein factors. Infiltration of the liver by the leukemic cells may "dilute" the potency of the liver cells as measured by biological assay. Mechanical or toxic injury due to infiltration may be inflicted upon the liver cells and check the storage of proteins in these cells.

Hypoproteinemia is viewed with disfavor by the clinician with good reason. That edema may develop needs no debate but there are degrees of hypoproteinemia not sufficient to produce edema yet adequate to lower the body defense against infection (4) and toxic liver injury (5). It is probable that such degrees of hypoproteinemia are more common than is generally appreciated. The drain coming from the fetus or lactation may cause such *plasma protein depletion* and therefore deserves the attention of the obstetrician. It should not be difficult by adequate diet to replete the protein reserve stores. In rare cases of vomiting plasma could be given by vein to replete these important stores. It is at least possible that one factor in precipitating the toxic condition designated *eclampsia* may be the serious depletion of body protein reserve stores. The severity of depletion of the protein stores may determine whether the clinical case of eclampsia does or does not present widespread hyaline liver necroses.

Hepatitis with jaundice often shows low stores of hemoglobin-producing factors—both iron and protein (Table 3). Low protein stores and hypoproteinemia favor liver injury—a vicious circle—as liver injury tends to slow up protein production. Obviously a correction of this state is greatly to be desired and if proteins cannot be given by mouth then plasma protein can be given by vein or peritoneum. Because methionine has a specific protective effect against

liver injury due to certain poisons (5), it deserves a clinical test where we suspect a continuing liver injury. Protein by mouth or plasma by vein should also furnish material needed for the prompt repair of injured liver or other tissues.

SUMMARY

Human liver tissue has been assayed to determine the amount of hemoglobin production factors in normal and abnormal states. Standardized dogs made anemic by blood removal have been used in this biological assay. Normal animal liver as control is rated as 100 per cent.

Normal human liver tissue as compared with the normal animal control contains more of these hemoglobin production factors—a biological assay ratio of 120 to 160 per cent. Infections, acute and chronic, do not appear to modify these values, the concentration of hemoglobin-producing factors falling within the normal range.

Pernicious anemia and aplastic anemia both show large liver stores of hemoglobin producing factors—a biological assay ratio of 200 to 240 per cent. Therapy in pernicious anemia reduces these liver stores as new red cells are formed.

Secondary anemia presents a low normal or subnormal liver store of hemoglobin producing factors—an assay of 60 to 130 per cent.

Hemochromatosis, erythroblastic anemia, and hemolytic icterus in spite of large iron deposits in the liver usually show a biological assay which is normal or close to normal.

Polycythemia shows low reserve stores of hemoglobin-producing factors. Leukemias present a wide range of values discussed above.

Hypoproteinemia almost always is associated with low reserve stores of hemoglobin producing factors in the liver—biological assays of 60 to 80 per cent. Hypoproteinemia means a depletion of body protein reserve stores including the labile protein liver reserves—a strong indication that the prehemoglobin material (or globin) is related to these liver stores.

Pregnancy, eclampsia, and lactation all may present subnormal liver stores of hemoglobin producing factors. Exhaustion of protein stores lowers the barrier to infection and renders the liver very susceptible to many toxic substances. It should not be difficult to correct hypoproteinemia under these conditions and thus relieve the patient of a real hazard.

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A GENERALIZED VISCERAL DISEASE OF GUINEA PIGS, ASSOCIATED WITH INTRANUCLEAR INCLUSIONS

By ALWIN M. PAPPENHEIMER, M.D., AND CHARLES A. SLANETZ, Ph.D.

(From the Departments of Pathology and of Animal Care, College of Physicians and Surgeons, Columbia University, New York)

PLATES 19 TO 22

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Amongst a small colony of guinea pigs being used for nutritional experiments, two have sickened with a disease characterized by interesting visceral lesions. Since further experimental study has been balked by our failure to transmit the disease to other animals, it seems worth while at this time to report upon the pathology. We have found no reference to a similar generalized disease of guinea pigs but the lesions are not unlike those which have been found by Goodpasture and Talbot (1) by Farber and Wolbach (2) and others in human infants, and by VonGlabn and Pappenheimer (3) in an adult.

The symptoms in the two affected animals were not in any way distinctive. We observed only a gradual loss of weight and appetite, lethargy, dullness of the eyes, and it was noted that the hair was easily shed. No temperature record was kept. One of the guinea pigs died spontaneously. The other was killed for study.

PROTOCOLS

Guinea pig 70 Female. Received September 17 1941. Weight 296 gm. Maintained on vitamin E-deficient diet V, supplemented by 10 cc. of tomato juice and 25 gm. of lettuce daily, plus 50 mg. of *dl*- α tocopherol acetate weekly. Normal 16 day cycles. 2 unsuccessful matings. Maximal weight of 585 gm. was attained on January 15, 1942, from which date the weight gradually declined to 525 gm. on April 1. The animal was found dead on April 8.

Autopsy Lungs—lower lobes congested with areas of hemorrhagic consolidation in upper lobes. Liver—large, yellowish, obviously fatty, with a few large areas of caseous necrosis visible on inferior surface. Gall bladder—contained colorless bile. On the serous surface, numerous elevated greyish pearly nodules about 1 mm. in size. Spleen—large and soft. Kidneys—swollen, slightly paler than normal. Ovaries—small. Uterus—not pregnant (last mating 9 days before death). Other organs normal in the gross.

Microscopic Examination.

Lungs—the septa are in places thickened by accumulations of cells, some of which are small lymphocytes but the majority large mononuclear elements with vesicular nuclei of irregularly oval shape. These resemble the epithelioid cells of early tubercles. The cellular agglomerations often form fairly circumscribed nodules centering about small blood vessels. Among the cells certain large elements are conspicuous

by virtue of their eosinophilic intranuclear inclusions, to be described in detail later. Masses of the epithelial cells, some with inclusions, entangled with coarse fibrin, partially fill certain alveoli. There are irregular patches of alveolar hemorrhage. The endothelial cells of some of the pulmonary veins are swollen and contain inclusions, but no inclusions are found within the epithelium of the contracted bronchi.

Liver—throughout the section, there are many large areas of necrosis, some of which are quite recent, since the outline of the liver cells is preserved and the liver cell nuclei, though shrunken and pyknotic, are still discernible (Fig 1). The remaining liver parenchyma is the seat of uniform fatty infiltration.

Intranuclear inclusions are found in the following situations: (1) within the epithelial cells of small bile ducts, (2) within large mononuclear cells or epithelioid cells in the portal connective tissue, (3) in large cells lying free within the portal veins, (4) rarely, within the nucleus of a liver cell.

Gall bladder—the lining epithelium is exfoliated in part, that which remains is normal. The submucosa is edematous, infiltrated with lymphoid cells, and contains many swollen fibroblasts. No definite inclusions are seen. The white nodules observed at the autopsy are composed of masses of epithelial glandular tissue which intercept the muscular bundles and project on the serosal surface. Mitoses are abundant, and the orientation of the cells is somewhat atypical, but there is no invasion of the stroma. In several of the gland cells are typical intranuclear inclusions.

Spleen—the normal appearance is greatly altered by the presence of large numbers of epithelioid cells with vesicular nuclei, many of which contain inclusions. These cells sometimes replace the central portion of the Malpighian follicles, surrounding the central artery, (Fig 2), sometimes they form irregular, poorly delimited masses within the pulp. Amongst these larger cells are pyknotic nuclear fragments from the destruction of the lymphocytes, also deposits of coarse fibrin. Inclusion-bearing cells are found both in the pulp and free within the splenic sinuses. Hemosiderin pigment is abundant.

Kidney—occasional individual epithelial cells of the glomerular capsule are swollen and contain inclusions. Many tubules are seen in which the lining cells are greatly swollen, and their nuclei distended with large inclusion bodies (Fig 3). The inclusion-bearing cells are often exfoliated, filling the lumen. Multinucleated cells are common, and in some of these, the nuclei are dark and the outline of the inclusion blurred. They are evidently degenerating (Fig 4). There is no diffuse interstitial reaction, but where the epithelial inclusions are most numerous, one finds occasional groups of epithelioid cells in the stroma, some of which also contain inclusions. No inclusion bodies are present in the collecting tubules or pelvic epithelium.

Myocardium—occasional inclusion-bearing mononuclears are found between the muscle fibers or in the vicinity of small branches of the coronary arteries.

Large intestine—normal. No inclusions. *Skeletal muscle*—normal. The salivary glands were not examined.

Guinea pig C-76. Female. Received February 16, 1942. Placed on diet V with daily supplement of 10 cc. of tomato juice and 25 gm. of lettuce, plus 10 mg. of dltocopherol weekly. Initial weight 210 gm. Maximal weight on April 8, 354 gm., followed by decline to 245 gm. when it was killed on April 20. No cycles.

Autopsy Moderate emaciation, hair falling out. Muscles dry, but of normal texture and color. Heart normal. Left ventricle contains loose grey clot. *Lungs*—normal. *Liver*—large, pale, yellowish brown with numerous areas of greyish white necrosis scattered through all lobes. *Spleen*—much enlarged congested, with scattered grey areas of necrosis (?). *Kidneys*—very pale, with greyish foci on surface and cut section. *Adrenals*—normal. *Ovaries*—immature. *Stomach*—mucosa diffusely thickened and edematous. Along lesser curvature, irregular superficial ulcers covered by necrotic greenish membrane. Other organs not abnormal.

Microscopic Examination.

Lungs—there are masses of pale epithelioid cells and lymphocytes about the smaller blood vessels extending into and thickening the adjacent septa. Amongst these are occasional clumps of coarse fibrin.

Cells with intranuclear inclusions are scattered amongst them, and occasionally larger elements sometimes with several nuclei, are found lying against the alveolar walls (Fig 5). The bronchial epithelium contains no inclusion bodies.

Myocardium—occasional large mononuclear cells with inclusions lie between the muscle fibers (Fig 6). There is little inflammatory reaction, in some areas, the inclusion bearing large mononuclears are accompanied by small lymphocytes. Occasionally a few muscle fibers in the vicinity of the inclusion-containing cells are necrotic.

Liver—there is moderate diffuse fat infiltration, as well as scattered areas of complete necrosis of liver cells, unaccompanied by any inflammatory reaction. Individual cells show hyaline necrosis. As in the previous case, inclusion bodies are found within (1) liver cells (Fig 7), (2) within the epithelium of the smallest bile ducts, (3) in epithelioid cells of the portal connective tissue, (4) in large mononuclear cells apparently circulating freely in the portal blood (Fig 8) or in portal lymphatics. Inclusions are not present within the epithelial cells of the larger bile ducts.

Spleen—the lesions are similar to those described in the previous case, but are even more striking. A large part of the splenic pulp consists of solid masses of pale staining epithelioid cells, sometimes with an interlacing fibrinous network, which have replaced the normal elements (Fig. 9). Amongst them are many multinucleated giant cells. Many of these cells contain large intranuclear inclusions (Fig 10). Lymphoid cells which are trapped in these areas are pyknotic and fragmented. The endothelium of the splenic sinuses rarely contains a small inclusion body. There is a moderate amount of hemosiderin pigment.

Lymph glands—(a) Beneath the capsule, there are irregular areas in which the normal structures are replaced by masses of pale staining epithelioid cells, many of which contain inclusions. There is fragmentation of the nuclei of the lymphocytes. (b) A second lymph node presents a remarkable appearance. The normal structure is profoundly altered—the lymphoid elements have been almost wholly replaced by large pale cells with bluish cytoplasm and vesicular nuclei. Many of the cells are multinucleated and eosinophilic inclusions are found in almost every nucleus. The outlines of the sinuses are not to be made out—there is hemorrhage and fibrin deposit. The pyknotic, broken up remains of the lymphocyte nuclei are scattered amongst the large cells. (c) A peripancreatic lymph node is the seat of similar extreme changes (Fig 11). Here many of the multinucleated plasmatic masses are undergoing de-

generation. The inclusion body is no longer separated from the nuclear membrane by a clear space, but fills the entire nucleus, which is shrunken and takes a reddish-purple stain with hematoxylin-eosin (Fig. 12).

Salivary glands—in some areas, swollen epithelial cells with large intranuclear, and also cytoplasmic inclusions, are abundant (Fig. 13). Both the duct and acinar cells are affected. There is a sparse lymphocytic reaction, the nuclei of the lymphocytes, as elsewhere, showing fragmentation. About some of the inclusion-containing ducts, there is no reaction whatever.

Pancreas—aside from interlobular edema, in part fibrinous, there are no lesions. *Adrenal*—normal. *Stomach*—over large areas, there is necrosis of the mucosa, extending almost or quite to the muscularis mucosae. In the necrotic tissue are masses of bacteria. At the base of the ulcerated areas, there are many pale fibroblasts, lymphocytes, often fragmented, and occasional polymorphonuclears. Some of the fibroblasts contain intranuclear inclusions. The submucosa is edematous (Fig. 14).

In other areas, the mucosa is intact and undergoing active proliferation. The epithelial cells are large and basophilic and mitoses are abundant. Some of the glands are dilated. In the vicinity of the necrotic areas, inclusion bodies are noted within the epithelial cells (Fig. 15). A large inclusion-bearing cell is found within a lymphatic vessel of the muscularis.

Small intestine—normal.

Uterus and Ovaries—normal. *Urinary bladder*—normal. *Parathyroid*—a single epithelial cell is found, containing an intranuclear inclusion. *Skeletal muscles*—normal. *Kidneys*—typical inclusions occur within hypertrophied cells of the convoluted tubules; these cells may be still attached to the basement membrane, or exfoliated into the lumen. Inclusions are seen also in isolated cells of the glomeruli. One glomerulus is composed almost wholly of swollen pale inclusion-bearing cells, whose origin, whether from capillary endothelium or epithelium, is uncertain. The capillaries themselves are compressed and empty. Small eosinophilic bodies, perhaps derived from the intranuclear masses by extrusion, are seen in the cytoplasm (Fig. 16).

Inclusions—these vary considerably in size and shape. They are sometimes spherical, but more often oblong and sometimes quite irregular in shape. Within a single nucleus, there may be, in addition to a large eosin-staining mass, several smaller clumps or spherules. With the stains used (hematoxylin-eosin, Laidlaw), no granular structure is demonstrable, but fine vacuoles are often present in the larger inclusions. The chromatin is pushed against the nuclear membrane, and the basic staining karosome is usually distinctly seen.

The cytoplasm of the affected cells stains a robin's egg blue and is faintly granular. It may contain one or several eosin-staining small spherical bodies.

Various stages in the degeneration of inclusion-bearing cells can be studied in the liver. The outline of the inclusion body becomes blurred, and the eosinophilic material may completely fill the nucleus, leaving only an indistinct margin of chromatin against the still intact membrane. In a more advanced stage, the nuclear membrane no longer stains. The cytoplasm becomes eosinophilic and hyaline, and the entire cell obviously necrotic.

As with other virus infections characterized by intranuclear inclusions, the affected cells undergo hypertrophy, and often become multinucleated. Giant cells, with

multiple inclusion-bearing nuclei, were particularly conspicuous in the lymph nodes and spleen.

The inclusions as has been shown, are present in many different cell types—in the epithelial cells of the salivary gland ducts, bile ducts, liver, kidney, gastric mucosa, and parathyroid, in fibroblasts, reticular cells, or histiocytes in endothelium, and even in circulating monocytes in the portal blood stream. We are familiar with no other virus inclusion having so catholic a distribution.

Bacteriological—cultures of kidney, liver, and spleen on defibrinated sheep blood agar plates, brilliant green agar and nutrient agar remained sterile. Negative cultures were also obtained from inoculated animals.

Attempts at Transmission

From the second guinea pig (C 76), kidney, spleen, and liver were removed aseptically, ground with sterile sand in saline, and a heavy suspension injected subcutaneously into 3 adult guinea pigs and 1 young one.

Daily temperatures and weights were recorded. The animals were sacrificed after 4, 7, 16, and 16 days. Guinea pig SI-4, injected with liver suspension, lost 53 gm. in 4 days and was killed. It was found to have extensive bronchopneumonia. A search for inclusion bodies in the lungs and other viscera was disappointing except for a bronchial lymph node. Here two large mononuclear cells with typical inclusions were found free within a sinus. In a strand of pale epithelioid cells about a portal space in the liver, another inclusion bearing cell was discovered. The characteristic visceral lesions of the spontaneous cases were lacking.

A liver suspension from SI-4 was injected into 2 other guinea pigs. One of them (SI 5) became ill, losing 35 gm. in weight in 7 days. No lesions or inclusion bodies were found on microscopic study. Guinea pig SI-6, killed after 12 days, showed no evidences of illness; there were no lesions or inclusion bodies. The salivary glands of both these animals were free of inclusions.

The 3 other guinea pigs injected with spleen and kidney from C 76 were negative so far as generalized lesions with inclusions were concerned. Only one showed a single inclusion body within a salivary gland duct epithelial cell.

Blood from SI-3 was injected into another animal and glycennated spleen and liver from C 76 were injected subcutaneously and intracerebrally into other guinea pigs and white mice. All these attempts at passage were fruitless.

DISCUSSION

We have described a generalized disease of guinea pigs, in which lesions of considerable severity were present in lungs, liver, spleen, kidneys, gastric mucosa, and lymph nodes. These were associated with conspicuous intranuclear inclusions of the type occurring in herpes, varicella, virus III, and many other virus diseases. Indeed, the appearance of the inclusion conforms in all details with that repeatedly described in the salivary gland virus infection of guinea pigs, and especially well pictured in the article of Rosenbusch and Lucas (4). The presence of these inclusions and the failure to demonstrate bacteria in the sections or in culture, make it highly probable that the disease is due to a virus.

The question at once arises as to whether one is dealing with a virulent form of the usually innocuous salivary gland virus, which is so often present in apparently healthy adult guinea pigs. The incidence of the salivary gland inclusions varies in different stocks, as Markham (5) has pointed out, but may be as high as 84 per cent (Cole and Kuttner, 6). In the studies which have been made of this disease, one can find no reference to generalized visceral lesions such as were present in our animals. Markham and Hudson injected the submaxillary gland virus into fetal guinea pigs, either intracerebrally or *via* the placenta. The latter route produced a wide dissemination of lesions and inclusion-bearing cells. They state specifically, however, that the inclusion bodies were always confined to the mesenchymal cells or tissues derived from them, in no instance were they found in epithelial cells (7). However, characteristic inclusions do occur in the renal epithelial cells (Jackson, 8).

The salivary gland virus, as was first shown by Cole and Kuttner, may be successfully transmitted to young guinea pigs by inoculation of infective salivary gland material. Intracerebral injection produces a meningitis with inclusion bodies, but serial transmission beyond one or two passages, has not been accomplished (9). Following inoculation of virus-bearing material directly into various viscera, inclusion-containing cells have been found in other situations than the salivary gland ducts—testis, tongue, and lung (Cole and Kuttner), and in fibroblasts, macrophages, endothelial cells of various viscera (4). While it is possible therefore experimentally to produce a generalization of the salivary gland virus, it should be emphasized that the spontaneous infection remains localized to the salivary gland, and renal epithelium.

While the salivary gland virus is readily transmitted to young animals, and may even cause a fatal disease (4), our efforts to transmit the infection either to young or old guinea pigs were unsuccessful. Could this have been due to an immunity conferred by previous spontaneous infection with the salivary gland virus? This is unlikely in view of the fact that one of the 4 injected animals contained no inclusions in the salivary gland, in another, only a single inclusion was found after thorough search. In the other two animals, the salivary glands unfortunately were not examined.

While the question of the identity of our agent with the salivary gland virus must remain open for the present, the evidence at hand argues against it.

Whatever the nature of the inciting agent, the natural infectivity of the disease appears to be slight. Animals kept in the same cage with the infected guinea pigs have remained well, and no epidemic has developed.

SUMMARY

A spontaneous generalized visceral disease of guinea pigs, characterized by the presence of intranuclear inclusion bodies of the herpes type, is described.

The possible relation of this disease to the salivary gland virus infection of guinea pigs is discussed

We wish to acknowledge with thanks the technical assistance of Claudia Schogoleff in the care of the animals and preparation of sections

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EXPLANATION OF PLATES

All the slides used for illustration are stained with hematoxylin and eosin

PLATE 19

FIG 1	Guinea pig C-70	Liver	Large areas of necrosis	× 230
FIG 2	Guinea pig C-70	Spleen	Large cell with intranuclear inclusion	× 460
FIG 3	Guinea pig C-70	Kidney	Intranuclear inclusions within epithelium of proximal convoluted tubule	× 460
FIG 4	Guinea pig C-70	Kidney	Degenerating giant cell	Several other cells contain inclusions
				× 460

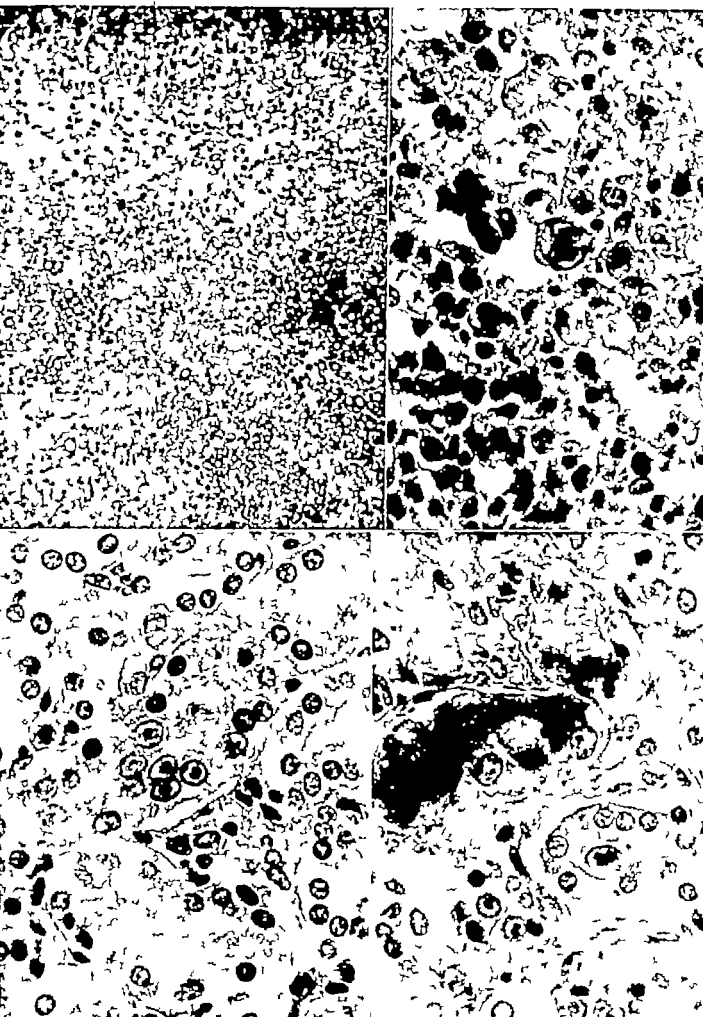


PLATE 20

FIG 5 Guinea pig C-76 Lung Large cells with intranuclear inclusions are seen lying against the alveolar septa $\times 460$

FIG 6 Guinea pig C-76 Myocardium Large inclusion bearing mononuclear cell between muscle fibers $\times 460$

FIG 7 Guinea pig C 76 Liver Inclusions within liver cells Necrosis and pigmentation of nuclei $\times 460$

FIG 8 Guinea pig C-76 Large inclusion-bearing mononuclears free in blood of a portal vein $\times 460$

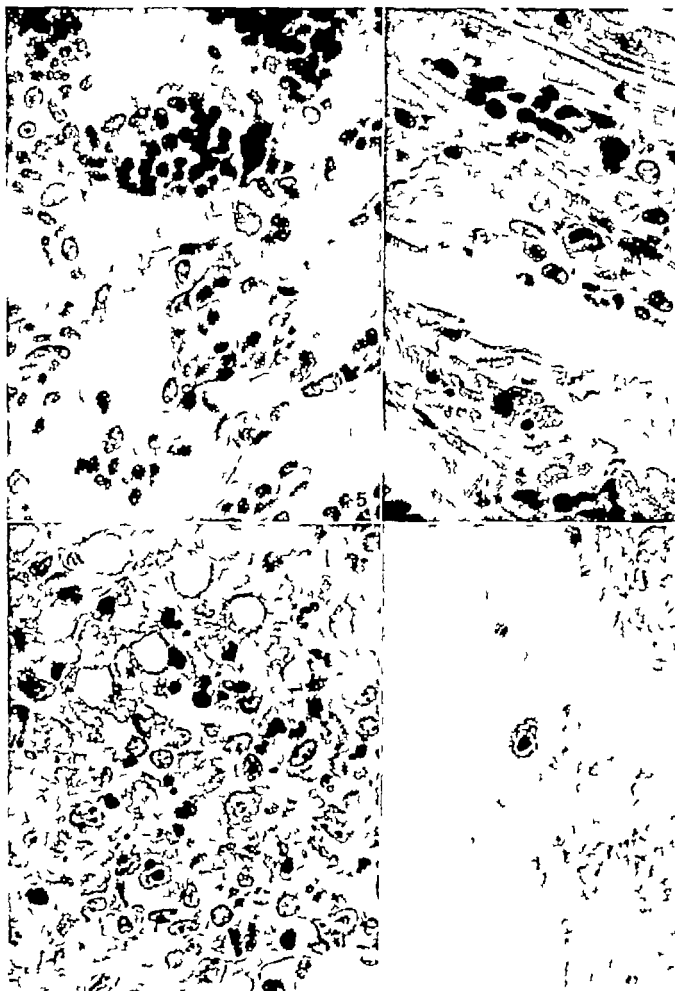


PLATE 21

FIG 9 Guinea pig C-76 Spleen Normal elements of pulp replaced by pale epithelioid cells Many giant cells $\times 230$

FIG 10 Guinea pig C-76 Spleen Intracellular inclusions and fragmentation of lymphoid cells $\times 460$

FIG 11 Guinea pig C-76 Peripancreatic lymph node Replacement of normal elements by large inclusion-bearing epithelioid cells Numerous giant cells, many of them degenerating $\times 230$

FIG 12 Guinea pig C-76 Peripancreatic lymph node Giant cells and intracellular inclusions $\times 460$

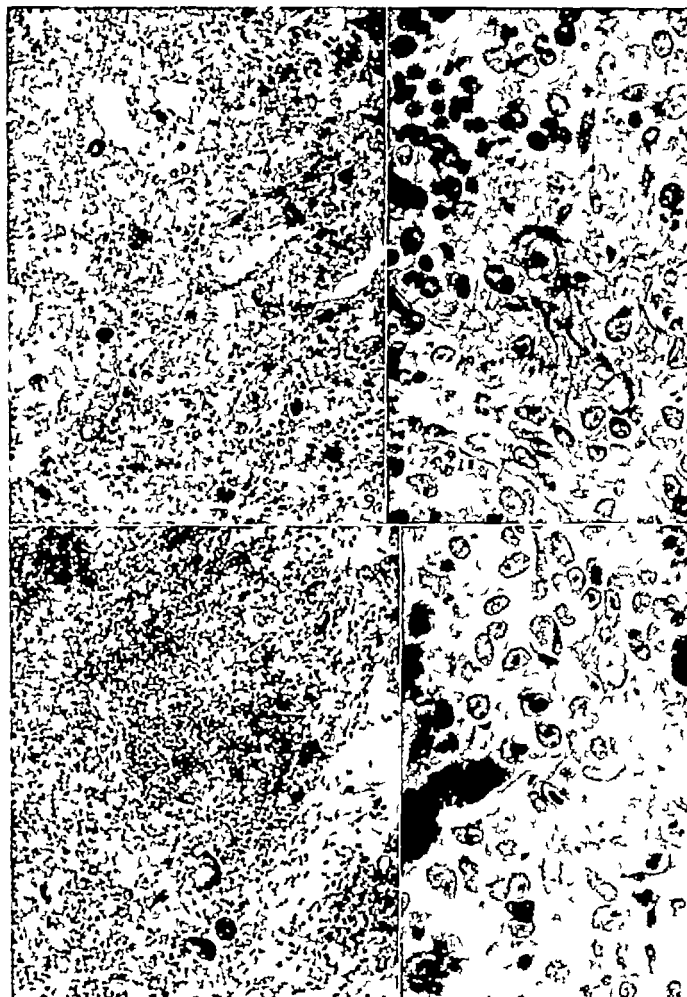


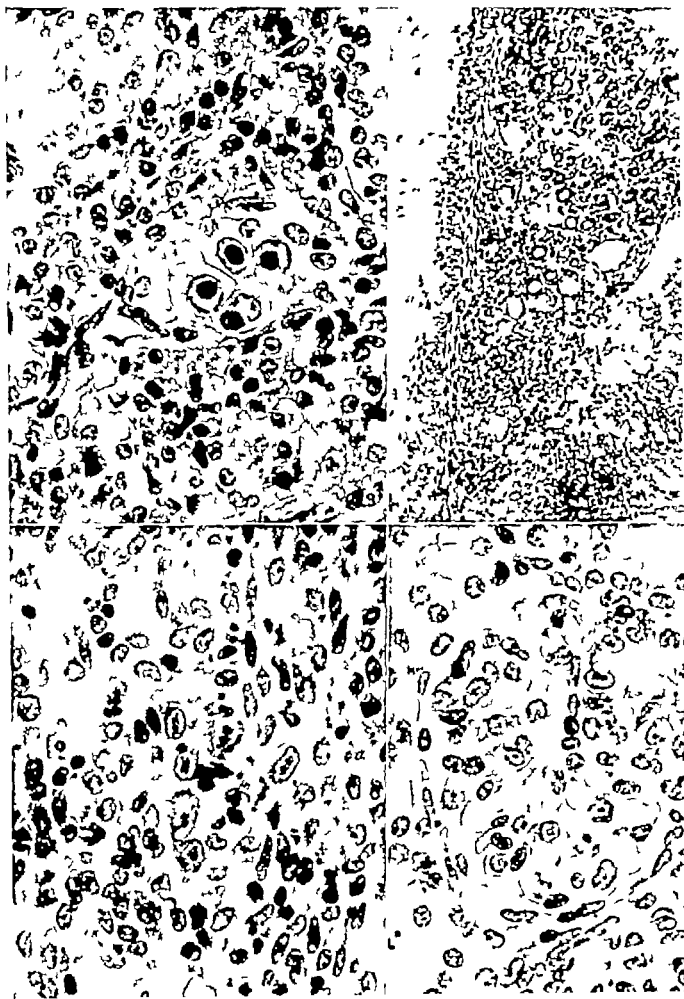
PLATE 22

FIG 13 Guinea pig C-76 Salivary gland Large inclusions in duct cells Lymphocytic reaction $\times 460$

FIG 14 Guinea pig C-76 Stomach Ulceration surmounted by necrotic slough $\times 110$

FIG 15 Guinea pig C-76 Stomach Inclusions in glandular epithelial cells in vicinity of necrotic area $\times 460$

FIG 16 Guinea pig C-76 Kidney Glomeruli, composed of pale swollen cells, most of which contain inclusions $\times 460$



STUDIES CONCERNING THE SITE OF RENIN FORMATION IN THE KIDNEY

III THE APPARENT SITE OF RENIN FORMATION IN THE TUBULES OF THE MESONEPHROS AND METANEPHROS OF THE HOG FETUS*

By A. KAPLAN Ph.D., AND MEYER FRIEDMAN, M.D

WITH THE TECHNICAL ASSISTANCE OF ELEANOR WILLIAMS

(From the Harold Brunn Institute for Cardiovascular Research, Mount Zion Hospital San Francisco)

PLATES 23 AND 24

(Received for publication, May 27, 1942)

In the first study (1) of the present series, the inability to detect a pressor substance in the aglomerular kidney of the midshipman fish, a marine variety, while abundant amounts of renin were demonstrated in the glomerular kidney of the catfish and carp, suggested that the site of renin production might be in the glomerular or juxtaglomerular component of the kidney. However, in subsequent studies (2, 3) it was found that no pressor substance, effective in the dog, could be found in the glomerular kidney of several other marine fish (cod, sole, mackerel) investigated in this laboratory. Bean (4), moreover, has recently reported the absence of renin in the kidney of the shark, a marine fish possessing a glomerular kidney. All observations thus point to the conclusion that the kidneys of marine fish, whether glomerular or aglomerular, are probably devoid of a renin like, pressor substance.

The failure of extracts of marine fish kidneys to exert a pressor effect when injected intravenously into dogs might possibly be due to the species specificity necessary for the reaction between renin and the activator (hypertensinogen?) in plasma as reported by Bean (4) and by Corcoran, Helmer, and Page (5). This possibility is unlikely, however, in view of the fact that extracts of kidneys of fresh water fish exhibit pressor properties typical of renin when injected intravenously into dogs. It should also be noted that Bean (4) was unable to detect the presence of renin in shark kidney by either (a) injecting shark kidney extract into other poikilotherms and thus eliminating the effect of species specificity or (b) by obtaining hypertension (angiotonin) after incubation of shark kidney extract with plasma.

In the present report, the results of a study of the renin content and histological structure of the mesonephros and metanephros of the developing hog fetus are given, for it was found that these two kidneys followed entirely different paths of development as the fetus increased in length. The mesonephros of

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In summary then, the histological study of the mesonephros of fetuses between the lengths of 17 and 100 mm indicated that there was a continuous and differential necrosis of the tubules, with the glomerular component remaining unchanged until metaplasia of the residual mesonephros occurred

Description of the Hog Metanephros

Gross Findings—The striking characteristic of the metanephros of the smallest fetuses studied (25 to 30 mm) was the relative paucity of mature tubular epithelium and the relatively heavy concentration of mature and immature glomeruli. The tubular epithelium (Fig 3) was for the most part poorly differentiated, rarely equipped with a brush border, and appeared basophilic on staining with hematoxylin and eosin. In the interior of the kidney, however, a few mature appearing tubules were seen, together with numerous large, lobulated glomeruli containing nucleated red blood cells. A good proportion of the metanephric tissue, however, was observed to be mesenchymal interstitial tissue. Some collecting duct epithelium was observed.

When the metanephric tissue of older fetuses (30 to 300 mm) was studied, it was observed that the growth of the kidney was confined chiefly to the tubular portion (Fig 4), particularly to the proximal convoluted tubules (as now identified by its brush border). In contrast to the tubular growth, there was a progressive diminution in the amount of the mesenchymal tissue and in the concentration of glomeruli as indicated by the number per low power field. As in the smaller fetuses, the principal site of both tubular and glomerular development was found to be in the peripheral sections of the kidney.

In metanephra of fetuses over 100 mm in length, a rapid growth and extension of collecting duct epithelium was observed, which extended from the beginning pelvis of the kidney and radiated outwards, toward the periphery.

Here again, extensive study of many sections of metanephra taken from fetuses varying from 20 to 300 mm in length failed to reveal any accumulation of cells in a juxtaglomerular position which could be considered secretory in function. As can be seen in Fig 5, the afferent glomerular arteriole invariably was found to be devoid of any specialized, granular cells. In view of these observations, it was concluded that the fetal metanephros lacked the juxtaglomerular accumulation of granular cells described by Goormaghtigh (7) in the kidney of the adult rabbit.

Complete transverse sections of metanephra taken from fetuses of varying length were examined for the number of mature glomeruli they contained per low power field (magnification, 100 times). The entire kidney section was divided into areas approximating the diameter of the low power field. The average number of glomeruli per low power field obtained in this manner was averaged with similar results obtained from other counts of metanephric tissue from fetuses of similar size. It was found that the metanephric kidney from fetuses of 25 to 49, 50 to 74, 75 to 99, 100 to 199, and 200 to 300 mm lengths,

contained an average of 14, 11, 9, 9, and 8 mature glomeruli respectively, per low power field, indicating that the number of glomeruli per gram of kidney tissue decreased with advancing fetal development

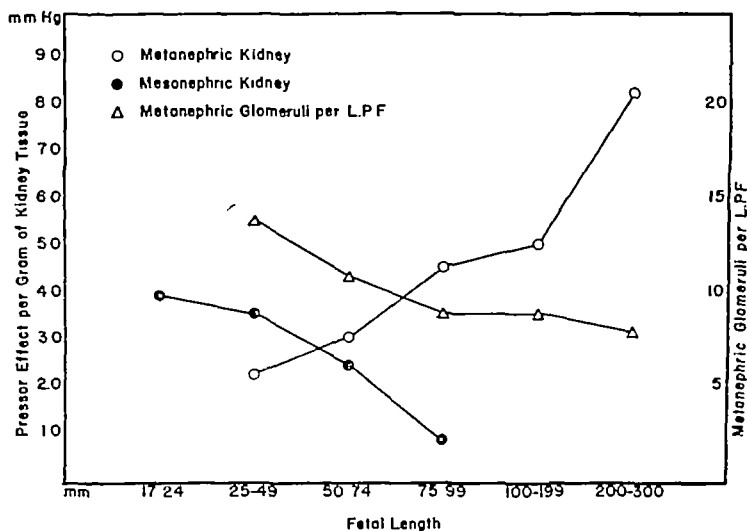
TABLE I
The Renin Content of Mesonephric Kidney at Various Stages of Fetal Growth

Length of fetus	Fetal kidney extract	Recipient dog	Extract injected (gm. fresh kidney)	Mean arterial blood pressure		Pressor effect (Rise per gm. kidney)
				Before injection	After injection of fetal kidney extract	
mm				mm Hg	mm Hg	mm Hg
17 to 24	21 S	42	5 0	144	160	3 2
	21 S	38	5 0	118	146	5 6
	149-S	12	5 0	128	148	4 0
	149-S	46	5 0	126	142	3 2
						Average 4 0
25 to 49	12 S	0	10 4	160	181	2 0
	12-S	0	8 3	130	160	3 6
	64-S	10	5 0	106	126	4 0
	64-S	38	5 0	130	146	3 2
	64-S	38	5 0	140	155	3 0
	100-S	23	5 0	155	178	4 6
	100-S	45	5 0	110	134	4 8
						Average 3 6
50 to 74	69-S	23	10 0	86	115	2 9
	69-S	19	10 0	145	164	1 9
	101-S	3	10 0	123	133	1 0
	101-S	42	10 0	152	170	1 8
	115-S	10	5 0	108	132	4 8
						Average 2 5
75 to 99	25-S	33	5 0	138	143	1 0
	25-S	42	5 0	148	148	0
	25-S	42	10 0	150	168	1 8
	25-S	38	5 0	150	152	0 4
	150-S	26	10 0	138	148	1 0
	150-S	10	12 0	104	116	1 0
						Average 0 86

The Amount of Pressor Substance in the Mesonephros

As can be seen in Table I and Text fig 1, the mesonephric tissue of the hog fetus contained a pressor substance effective in the dog which decreased in potency per gram of fresh tissue as the length of the fetus increased. Thus, it

was found that two extracts, obtained from the mesonephra of fetuses between 17 and 24 mm in length when tested four times on four dogs, caused an average rise in blood pressure of 4.0 mm Hg per gm of fresh mesonephros. The average rise in blood pressure following the injection of three different extracts obtained from mesonephra of fetuses between 25 and 49 mm in length was found to be 3.6 mm Hg per gm of fresh mesonephros. Three extracts obtained from the mesonephra of fetuses between 50 and 74 mm in length, tested five times on five dogs, effected an average rise in blood pressure of 2.5 mm Hg, while the two extracts obtained from the degenerating mesonephra of fetuses



TEXT-FIG 1 The pressor effect of mesonephric and metanephric extracts from hog fetuses of increasing length upon the dog. Also the relation of metanephric glomeruli per low power field ($\times 100$) to the size of the fetus

between 75 and 99 mm in length, effected an average rise in blood pressure of but 0.86 mm Hg per gm of fresh mesonephros

The Amount of Pressor Substance in the Metanephros

All metanephric kidneys contained a pressor substance in good amount, whatever the length of the fetus from which they came. The concentration increased with the age of the fetus as is not the case in the mesonephros. Thus, an extract obtained from metanephra of fetuses from 25 to 49 mm in length (Text-fig 1 and Table II), when tested four times on three dogs, effected an average blood pressure rise of but 2.3 mm Hg per gm of fresh metanephros, whereas five extracts obtained from the metanephra of fetuses varying from

TABLE II
The Renin Content of Metanephric Kidney at Various Stages of Fetal Growth

Length of fetus	Fetal kidney extract	Recipient dog	Extract injected (gm. fresh kidney)	Mean arterial blood pressure		Pressor effect (Rise per gm. kidney)
				Before injection	After injection of fetal kidney extract	
mm				mm. Hg	mm Hg	mm Hg
25 to 49	23-T	2	4.9	124	136	2.5
	23-T	45	4.9	128	140	2.5
	23-T	45	4.9	140	150	2.0
	23-T	38	4.9	132	142	2.0
						Average 2.3
50 to 74	43-T	0	15.9	132	154	1.4
	91-T	3	5.0	112	126	2.8
	91-T	42	5.0	154	166	2.4
	50-T	75	7.8	145	165	2.6
	65-T	21	10.0	140	164	2.4
	115-T	4	5.0	148	170	4.4
						Average 3.1
75 to 99	1	17	4.4	125	155	6.8
	1	16	5.5	120	138	3.3
	1	18	8.8	130	180	5.7
	\-6	9	10.0	156	194	3.8
	66-T	9	10.0	130	166	3.6
						Average 4.6
100 to 199	6	7	5.5	135	169	6.1
	22	13	5.5	122	148	4.7
	22	14	5.5	140	165	4.6
	94-T	3	5.0	94	126	6.4
	147-T	24	5.0	125	144	3.8
	147-T	00	5.0	150	170	4.0
	147-T	0	5.0	148	187	7.8
	147-T	26	5.0	127	151	4.8
	155-T	94	5.0	140	160	4.0
						Average 5.1
200 to 300	95-T	3	5.0	123	143	4.0
	95-T	3	5.0	128	148	4.0
	95-T	42	5.0	142	178	7.2
	148-T	10	5.0	130	190	12.0
	148-T	38	2.5	134	164	12.0
	142-T	26	5.0	123	175	10.4
	142-T	24	5.0	118	166	9.6
	142-T	94	5.0	137	175	7.6
	X-T	94	5.0	123	158	7.0
	\-T	26	5.0	136	188	10.4
	\-T	24	5.0	128	180	10.4
	160-A	26	5.0	114	190	15.2
	160-A	26	5.0	132	172	8.0
	160-B	21	5.0	148	176	5.6
	160-B	46	5.0	130	164	6.8
	171-T	12	5.0	138	172	6.8
	171-T	26	3.2	134	150	5.0
						Average 8.3

50 to 74 mm in length, when tested six times on six dogs, effected an average blood pressure rise of 3.1 mm Hg per gm of fresh tissue. Further, it was found that three extracts obtained from the metanephros of fetuses from 75 to 99 mm in length caused an average rise of 4.6 mm of Hg per gm of fresh metanephros in the blood pressure of the four dogs tested. This progressive increase in the concentration of pressor substance continued with the growth of the fetus. Five extracts obtained from the metanephros of fetuses from 100 to 199 mm in length were tested on nine dogs and it was observed that a rise in blood pressure of 5.1 mm Hg per gm of fresh metanephros was the average result. Finally, the average rise in blood pressure obtained by the injection of 10 dogs with 17 injections of seven different extracts obtained from the metanephros of fetuses varying from 200 to 300 mm was 8.3 mm Hg per gm of fresh metanephros. This latter potency closely approached that of the adult hog kidney cortex, which was found to be 8.8 mm Hg per gm of fresh kidney cortex.

The Identification of the Pressor Substance Found in Fetal Renal Tissue As Renin

The pressor substance found in both the mesonephric and metanephric tissue of the developing fetus appeared to be identical with renin, whatever the length of the fetus. Extracts obtained from mesonephric tissue of fetuses varying from 17 to 75 mm in length and from metanephros of fetuses from 25 to 300 mm in length exhibited a pressor effect which, (a) was uniformly slow in beginning but lasted over 10 minutes, (b) was not inhibited by the prior intravenous administration of cocaine, and (c) was abolished by the prior establishment of a tachyphylaxis to known hog renin in the recipient dog. Conversely, it was established that dogs which had received repeated injections of these fetal extracts became tachyphylactic to further injections of fetal extracts and to injections of known hog renin.

DISCUSSION

The degeneration of the mesonephros and the growth of the metanephros in the developing hog fetus were found to be characterized by differential degeneration of the tubular component in the former and its growth in the latter type of kidney. The number of glomeruli of the mesonephros remained unchanged despite the tubular decay, whereas those of the metanephros decreased per unit area as tubular proliferation went on. Furthermore, the amount of renin extracted from these two types of kidney, was found to decrease with tubular necrosis and to increase with tubular hyperplasia, despite the condition or number of functioning glomeruli present. Thus, the effective renin content per gram of tissue decreased nearly fourfold in the degenerating mesonephros at the same time that the number and function of glomeruli did not change, and conversely the renin content per gram of tissue increased nearly fourfold in the developing metanephros at the same time that the number of glomeruli

per unit area (and presumably per unit weight) decreased 40 per cent. This indicated clearly that the production of renin was independent of the arteriolo-glomerular component of the fetal mammalian kidney and varied directly with tubular mass

Since the results of our present studies indicate that the tubular epithelium of both mesonephros and metanephros is responsible for the formation of renin, it is interesting to speculate upon which section of the tubule is directly concerned. The presence of renin in both the metanephros and mesonephros and the absence of the loop of Henle and extensive collecting duct epithelium in the latter tissue suggest that these portions of the tubules are not responsible for renin production. Eliminating the loop of Henle and collecting duct epithelium by this consideration, only the convoluted tubular epithelium remains as the probable site of formation of renin. It should be noted that it was precisely this type of epithelium which was observed to increase differentially in the growing metanephros. At this time, however, it is still uncertain which portion of the convoluted tubules, the proximal or distal or both, is involved in the formation of renin.

CONCLUSIONS

1 Renin was found in both the mesonephric and metanephric kidneys of the smallest hog fetuses examined. These were from 17 to 24 mm. in length in the case of the former, and 25 to 49 mm. in that of the latter.

2 No evidence was found in either type of kidney of juxtaglomerular cells described by Goormaghtigh as the probable site of renin formation.

3 The renin content in both the mesonephros and the metanephros was found to be independent of its arteriologlomerular component but directly dependent upon the number, size, and functional state of the tubular component. It increased in amount with increasing tubular proliferation during the course of embryonic development, and decreased with the progressive tubular atrophy and degeneration incident thereto.

4 The site of renin formation is discussed.

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EXPLANATION OF PLATES

PLATE 23

FIG 1 The mesonephros of a hog fetus, 20 mm in length Note the large glomerulus which contains nucleated red blood cells The tubular epithelium appears in good condition and of a cuboidal type Compare tubules with those shown in Fig 2 Hematoxylin and eosin $\times 200$

FIG 2 The mesonephros of a hog fetus, 66 mm in length In this section, necrotic tubular epithelium, intact, red-blood-cell-containing glomeruli, and invading epigenitalis (upper right) can be seen The glomeruli do not disappear until all tubular epithelium has been replaced by this latter type of tissue Hematoxylin and eosin $\times 100$

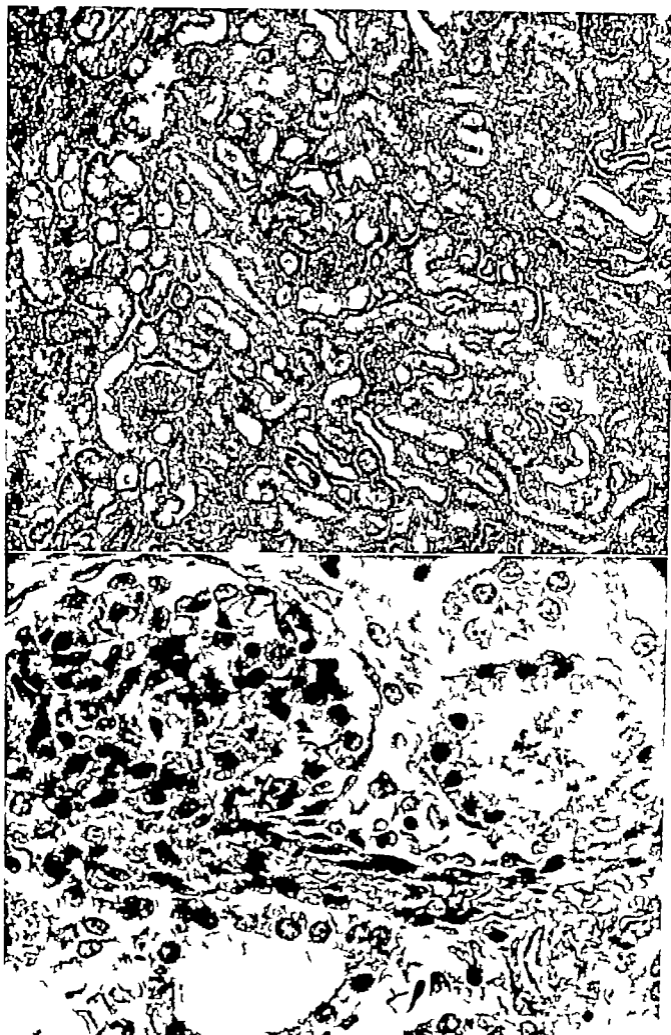
FIG 3 The metanephros of a hog fetus, 26 mm in length Note the large number of glomeruli, both mature and immature, as compared with the immature tubular epithelium widely separated by mesenchymal tissue Collecting duct epithelium can also be seen Hematoxylin and eosin $\times 100$



PLATE 24

FIG 4 The metanephros of a hog fetus, 175 mm in length. Note the abundance of mature appearing tubular epithelium and the decrease in mesenchymal tissue and glomeruli in this section. Hematoxylin and eosin $\times 100$

FIG 5 The glomerular afferent arteriole in the metanephros of a hog fetus, 66 mm in length. It is seen to be devoid of any type of cell other than the simple, flat cells composing its wall. Red blood cells can be seen both in the arteriole and in the glomerulus. Hematoxylin and eosin $\times 400$



These 3 compounds, *L*-cystine, cysteic acid, and taurine possess an amino group separated from a sulfur molecule by a 2 carbon chain. If the liver lesions caused by cystine and cysteic acid were due to this structural similarity, taurine should also produce the liver lesions.

The present experiments attempt to define the structural relation of the cystine sulfur to the production of liver lesions. The general behavior and the lesions noted in rats fed large amounts of cysteic acid, *DL*-methionine, and taurine are described.

Methods

Albino rats of both sexes and various ages were employed. Although previous cystine studies (4, 5) were carried out on 6 week old male rats, unpublished observations indicate that cystine liver damage may be produced in rats regardless of age or sex.

Cysteic acid was made from *L*-cystine by the bromine oxidation method and was fed as the sodium salt. The *DL*-methionine and taurine were obtained from the Eastman Kodak Company. The sulfur content of 15 per cent cysteic acid is approximately equal to that of 10 per cent cystine. Cysteic acid, in a concentration of 1.25 to 15 per cent in the McCollum stock diet, was fed to 15 rats. The food intake of each rat was restricted to 8 gm. a day. 6.4 to 12.4 per cent *DL*-methionine was added to the McCollum stock diet and fed to 26 rats. 12.4 per cent *DL*-methionine contains the same amount of sulfur as 10 per cent cystine. The daily food consumption of only 2 of the *DL*-methionine fed rats was measured and averaged 3.1 gm. a day, considerably below normal. The food consumption of the remaining rats also appeared to be low. Taurine was fed as 1 to 10 per cent of the McCollum stock diet to 130 rats. The daily food consumption was restricted to 10 gm.

Tissues from each rat were obtained at intervals after the onset of the feeding experiment and were fixed in Zenker's fluid and 10 per cent formalin.

Liver fat was measured in some of the methionine experiments by an ether extraction method (4).

RESULTS

CYSTEIC ACID—Five rats were fed cysteic acid as 1.25 per cent of the diet, 5 were fed cysteic acid as 6.25 per cent of the diet, 2 as 12.5 per cent of the diet, and 3 as 15 per cent of the diet. All animals were sacrificed after 8 to 14 days of this feeding.

General Condition—None of the rats fed cysteic acid died and all appeared to be in excellent condition at the time of sacrifice. They each received 8 gm. of food daily. The 10 rats fed 1.25 or 6.25 per cent cysteic acid maintained their weight. The 5 rats fed 12.5 to 15 per cent cysteic acid lost 7 to 43 gm. Five control rats fed 8 gm. *per diem* of the McCollum stock diet without cysteic acid lost 2 to 29 gm. in the same time.

Pathological Findings—When the concentration of cysteic acid in the diet was

6.25 per cent or less, it produced no anatomical changes. However, 12.5 to 15 per cent cysteic acid produced liver lesions in all 5 rats. The lesions resembled those described as due to *L*-cystine (4), particularly when 10 per cent *L*-cystine was fed in the McCollum stock diet (5). There was some portal necrosis of liver cells with varying degrees of portal fibrosis. In 2 instances the cirrhosis was evident from the gross appearance. The livers of these 2 rats had finely granular capsules and the cut surfaces showed fine intercommunicating scars confined to the portal areas. A conspicuous microscopic feature was the extensive proliferation of bile ducts in some of the scarred areas (Fig 1). The livers of the other 3 rats showed no gross changes. Histological examination, however, revealed numerous scarred portal areas with some proliferation of bile ducts. The portal scarring appeared to be due to both condensation fibrosis and connective tissue proliferation. The lack of changes visible in the gross in these 3 instances was due to the fact that the portal scarring showed little intercommunication with adjacent areas or extension into surrounding parenchyma. The liver cells were often hypertrophied but they showed little vacuolization and no fatty infiltration. A noteworthy trait was the absence of hemorrhage.

DL METHIONINE —A total of 26 rats were fed excess amounts of *DL* methionine. One rat received *DL* methionine as 6.4 per cent of the diet while all the other rats were fed 10 or 12.4 per cent *DL* methionine.

General Condition. Unlike the rats fed large amounts of cysteic acid, the rats fed *DL* methionine ate poorly and after several days appeared ill. They all lost weight rapidly averaging 5.0 gm a day. This was equivalent to a daily decline of 5.6 per cent of the original body weight. One rat lost 47 per cent of its original body weight in 13 days.

There was a considerable mortality among the rats fed excess *DL* methionine. Disregarding the 14 rats sacrificed during the first 5 days, 8 of the remaining 12 rats were dead by the end of 1 week. One rat lived 17 days and was then sacrificed. Three of the 4 rats that survived more than 7 days were adults, all other rats in this experiment being 6 to 8 weeks of age.

Pathological Findings. The rats usually showed evidence of severe weight loss in the diminution or absence of subcutaneous and mesenteric fat and in the moderate to severe dehydration. The liver and spleen almost invariably were decreased in size and had sharp edges. The liver capsule was smooth, clear, and transparent and the organ had a dark brown color. In 2 instances the edges of the liver were greyish yellow in color and had a translucent appearance. This translucent portion was wedge-shaped on cross-section with the base of the wedge away from the liver margin (Fig 2. This photograph shows the liver of a rat fed 10 per cent *DL* methionine for 4 days.) The liver parenchyma was very friable.

The essential change in the liver was the extreme atrophy of the liver cells

noted in 24 of the 26 rats fed excess *dl*-methionine. The diameter of the cells was much reduced and the cytoplasm was very dense and granular. The nuclei were also reduced in size, although this shrinkage was relatively less than that of the cells. The nuclei showed changes in the distribution of the chromatin which was condensed and often deposited at the periphery of the nucleus. The central nucleolus was more conspicuous than usual. The space between the nucleolus and the nuclear wall had very little stainable material. Fig 3 is a photograph of atrophic liver cells of a *dl*-methionine fed rat. For comparison, Fig 4 shows the same magnification of the normal liver cells of a rat fed the same McCollum stock diet without the *dl*-methionine supplement. The greyish yellow translucent parenchyma seen in the fresh liver in 2 instances showed much more severe atrophy than was seen elsewhere. No necrosis or other degenerative changes were evident, even in these areas. In one rat, a few isolated necrotic cells with hyalinized cytoplasm and dense pyknotic nuclei were scattered throughout the liver without any relationship to the lobular structure. No fibrosis, bile duct proliferation, or jaundice was found. The liver fat was much reduced in 7 methionine fed rats sacrificed after 4 to 6 days of feeding. It varied from 0.7 to 1.6 per cent of the whole fresh liver substance with an average of 1.2 per cent, in contrast to the livers of 12 control normal rats which had a range of 3.0 to 4.5 per cent (5).

An attempt to quantitate the degree of liver atrophy was made by counting with a microscope the number of portal spaces in 10 consecutive fields. At a magnification of 100 the average number of portal areas in 7 normal livers ranged from 1.2 to 3.6 per field with a mean of 2.2. In the 26 methionine fed rats the average number of portal areas ranged from 1.4 to 8.9, with a mean of 4.7. Of these, the 2 normal appearing livers of the methionine fed rats had average numbers of portal areas of 1.4 and 2.6 respectively. The remainder ranged from 3.1 to 8.9. One obvious source of error in the evaluation of the number of portal areas is the degree of distention of the liver sinusoids. In spite of this, however, the number of portal spaces in a unit area was much greater in the livers of the *dl*-methionine fed rats than in the rats fed the control McCollum stock diet.

The spleen showed microscopic evidence of atrophy, namely decrease in size of the Malpighian bodies, decrease in the number of cells in the pulp, and condensation of the pulp structures in 15 of the 22 cases examined. Hemorrhage was present in the pulp of one spleen and congestion of the sinusoids was prominent in another.

The kidneys were atrophied in only 3 of the 26 cases. These 3 instances occurred in rats showing the most extreme liver atrophy, the average number of portal areas in these livers being 6.3, 8.0, and 8.9 per low power field respectively. Dilatation of the convoluted and collecting tubules was the most usual alteration in the kidneys and occurred in half the *dl*-methionine fed rats.

The epithelial cells of these tubules were small, vacuolated, and had a basophilic cytoplasm. In one case, several mitotic nuclei were found among these vacuolated epithelial cells. Hyaline droplets were not seen in these cells. The glomeruli were not altered.

In 3 instances the stomach had submucosal hemorrhages in the antral portion beneath the squamous cell lining. The fundus showed no changes.

The lungs showed no specific lesions. Heart, testes, and pancreas were normal.

The results with feeding *dl*-methionine, as far as studied, were not influenced by the diet in which the *dl*-methionine was administered. It has been found that *l*-cystine fed in a low protein, low fat diet produced more severe liver lesions than when fed in the McCollum stock diet (5). For this reason, 5 rats were fed 10 per cent methionine in the same low protein, low fat diet. This diet consisted of 5 parts of casein, 3 of lard, 2 of cod liver oil, 5 of brewer's yeast, 4 of salt mixture, 10 of *dl* methionine, and 71 of sucrose. This low protein, low fat diet did not influence the effect of the 10 per cent methionine.

TAURINE—Rats weighing 80 to 100 gm. were fed excess amounts of taurine. This was fed as follows: 29 females—1 per cent taurine for 6 weeks, 68 females—2.5 per cent taurine for 8 weeks, 10 males—10 per cent taurine for 7 weeks, 20 females—stock diet without taurine for 7 weeks. The food intake was restricted to 10 gm. daily.

General Condition The rats ate all their food and grew as well as their controls.

Pathological Findings There were none in any of the organs examined which included liver, spleen, kidney, heart, aorta, lung, suprarenal, pituitary, thyroid, ovaries, testis, uterus, and brain.

DISCUSSION

The above data show that excess dietary cysteic acid produces portal necrosis and cirrhosis similar to that caused by comparable amounts of dietary *l*-cystine (4, 5). The ingestion of similar amounts of methionine or taurine did not produce such liver lesions. Urinary sulfate is formed in large amounts from *l*-cystine and *dl* methionine feeding but not from cysteic acid (5). Since feeding *l*-cystine or cysteic acid produces liver necrosis and cirrhosis these lesions are not dependent on either the S-S grouping of cystine, the degree of oxidation of the sulfur in the cysteic acid, the formation or excretion of urinary sulfate which occurs with *l*-cystine and *dl*-methionine but not with cysteic acid, or the presence of the amino group and S molecules separated by a 2 carbon chain in the cystine, cysteic acid, or taurine.

The general reaction of rats fed 1.25 to 15 per cent cysteic acid differed from those fed similar quantities of cystine. Rats fed large amounts of cysteic acid ate 8 gm. of food daily while those receiving similar concentrations of *l*-cystine

ate only about 3 gm daily (5) Those fed 12.5 to 15 per cent cysteic acid did not appear ill and none died within the 2 weeks during which they were studied In contrast, the mortality during the first 2 weeks of 10 per cent *l*-cystine feeding varied from 71 to 100 per cent depending on the basal diet (5)

The atrophy of the liver caused by feeding excess amounts of *dl*-methionine is a most striking change Although the food intake of the *dl*-methionine fed rats was not measured, the atrophy was certainly not due to starvation alone, since it was present in the livers of rats fed the *dl*-methionine for only 2 days In the two instances in which the food intake was measured it was 3.1 per 100 gm body weight daily Six rats weighing about 100 gm were fed 3.0 gm of the stock diet for 2 to 6 days and showed no such atrophy The severe general dehydration may have played some part in shrinking the liver cells, but if so, this effect appears to have been relatively specific for the liver since it occurred less frequently in the spleen and rarely in the kidney *dl*-Methionine is acted on directly by liver cells (10) Unpublished observations show that the excised livers of *dl*-methionine fed rats have an increased metabolism as compared with livers fed the stock diet alone The liver atrophy may be a reaction to this increased metabolism

In spite of the usual severe atrophy of the liver and spleen, and occasional atrophy of the kidney, there is no apparent anatomical lesion that offers an explanation for the fatal outcome due to feeding excess *dl*-methionine

SUMMARY AND CONCLUSIONS

1 Cysteic acid fed to albino rats as 12.5 to 15 per cent of the McCollum stock diet caused portal necrosis and cirrhosis of the liver within 2 weeks Concentrations of cysteic acid of 6.25 per cent or less in the diet produced no liver lesions within 2 weeks

2 *dl*-Methionine fed as 6.4 to 12.4 per cent of the McCollum stock diet or of a low protein, low fat diet, resulted in severe atrophy of the liver cells but no cirrhosis of the liver

3 Taurine fed as 1 to 10 per cent of the McCollum stock diet produces no liver lesions

4 For reasons discussed in the paper, it is concluded that the liver necrosis and cirrhosis produced by cystine and cysteic acid are not dependent upon the S-S linkage of the cystine, the oxidation of the sulfur, the formation and excretion of large amounts of urinary sulfate, or the presence of an amino group separated from a sulfur molecule by a 2 carbon chain

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EXPLANATION OF PLATE 25

FIG 1 Cirrhosis of the liver following ingestion of 15 per cent cysteic acid for 8 days. Fibroblast and bile duct proliferation in the portal areas with relatively slight liver cell necrosis. Hematoxylin and eosin stain. Magnification $\times 100$.

FIG 2 Liver atrophy in rat fed 10 per cent *dl*-methionine for 4 days. The edges appearing as light grey in the photograph were yellow in the fresh organ, while the remainder was brown. The white areas on the surface of the liver are reflections from the light source. Natural size.

FIG 3 Atrophy of liver cells in rat fed 10 per cent *dl*-methionine in McCollum stock diet for 4 days. Hematoxylin and eosin stain. Magnification $\times 460$.

FIG 4 Normal liver cells of rat fed McCollum stock diet. Hematoxylin and eosin stain. Magnification $\times 460$.

(Earle *et al* Relation of cystine sulfur to liver lesions)

STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

VI. FACILITATION OF INFECTION IN THE MOUSE*

BY LESTER S. KING M.D.

(From the Laboratory of the Fairfield State Hospital, Newtown, Connecticut, and the Department of Pathology, Yale University School of Medicine New Haven, Connecticut)

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Fixed strains of neurotropic viruses are characterized by relative inability to enter the central nervous system after peripheral inoculation. In previous communications (1) it was emphasized that a fixed strain of equine encephalomyelitis virus had very weak invasive powers when injected subcutaneously, intramuscularly, or intravenously. The incidence of infection after intramuscular inoculation could be sharply increased by the injection of 50 per cent glycerine intraperitoneally in adequate amounts (1). The present paper attempts to determine under what conditions this phenomenon is operative, whether it can be produced by other agents, and to gain some suggestions as to the mechanism involved.

Methods

In the present study the same strain of fixed E.E. virus used previously was employed. The material used for inoculations was infected mouse brain tissue emulsified 1:10 in sterile buffered salt solution, and centrifuged to throw down the coarse particles. This stock suspension (10^7) or appropriate decimal dilution was used. For propagation of the virus albino mice of the Rockefeller Institute strain, the same as that employed in previous studies, was employed. For experimental animals, Swiss mice, purchased in uniform lots from a single dealer, were used. These animals were all 12 to 13 weeks of age and had an average weight of about 22 gm. Their susceptibility to the virus determined by titration was no less than that of the strain used for previous publications. In all experiments in which virus was given intramuscularly, a standard dose of 0.25 cc. of suitable dilution was employed.

Facilitation Effect

In the original experiments leading to the observation of the facilitation effect, the 50 per cent glycerine was injected intraperitoneally. However the same effect could be produced if glycerine were given intramuscularly, as illustrated in the first experiment in Table I. The dosage given is of considerable importance. It was previously shown (1 a) that too small doses had no effect whatever on the action of the virus. The optimal dose, as shown

* Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

in Experiment 2, Table I, was found to be 0.30 to 0.35 cc of a 50 per cent solution. Larger amounts resulted in a too great immediate mortality, that is, the injected animals died within a few hours, indicated in the tables by the symbol X.

It was of interest to determine the maximal dilution of virus with which this effect could be obtained. As seen in Table I, Experiment 3, in the control group only a small percentage died after the undiluted (or 10^0) suspension.

TABLE I

Experiment No	Route of glycerine administration	Dose of glycerine	Dilution of virus suspension	Results
1	Intraperitoneal	0.25	10^0	3, 3, 3, 3, 4, 4, 4, X, X, X, X
	Intramuscular	0.25		2, 3, 3, 3, 4, 4, 4, 0
	None—control	None		0, 0, 0, 0, 0, 0, 0, 0
2	Intramuscular	0.30	10^0	2, 3, 3, 3, 3, 3, 3, 4, 4, 5
	Intramuscular	0.40		3, 3, 3, 4, X, X, X, X, X, X
	None—control	None		4, 6, 0, 0, 0, 0, 0, 0, 0, 0
3	Intramuscular	0.30	10^0	3, 3, 3, 3, 3, 4, 0, 0, 0, 0
	None—control			3, 4, 0, 0, 0, 0, 0, 0, 0, 0
	Intramuscular		10^{-1}	2, 3, 3, 3, 3, 4, 5, 0, 0, 0
	None—control			0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	Intramuscular		10^{-2}	2, 3, 3, 0, 0, 0, 0, 0, 0, 0
	None—control			N T

2, 3, = mouse died within 48, 72 hours after inoculation (etc.)

0 = mouse survived

X = mouse died within a few hours after inoculation. Death not due to virus action

N T = not tested

None at all died with the next higher decimal dilution. Animals receiving glycerine showed a significant mortality rate with suspensions of 10^0 and 10^{-1} , but in the next higher dilution the mortality was low, comparable to the 10^0 control group. In quantitative terms it can therefore be said that the glycerine treatment increased the effective titer not more than 100 times.

Mice after receiving glycerine show a fairly definite picture, consisting first of restlessness, followed by a period of apathy in which they sit with markedly ruffled fur. This stage is generally succeeded by definite prostration, interrupted by convulsive jerks and twitchings and sometimes frank generalized convulsions. Some animals succumb in this period but the others gradually recover. Those that survive 18 hours are indistinguishable from normal animals until the action of the virus makes itself

manifest Too small a dose of glycerine not leading to obvious signs, does not facilitate virus action.

When the glycerine is injected into one of the hind legs a great edematous swelling of the affected leg occurs in the course of $\frac{1}{2}$ to 1 hour Some measure of the changes going on within the animal was sought. Since the edema clearly indicated a redistribution of fluid, attempts were made to excise in symmetrical fashion the edematous and control legs and by weighing determine the amount of the fluid exudations. After numerous trials however, this method was discarded as not sufficiently accurate for use in such a small animal.

Blood Changes

Since the variations in the red blood count have yielded valuable information in numerous experimental studies on shock, large numbers of determinations were made on mice but this method too was not satisfactory Blood was drawn from the tail

SPECIFIC GRAVITY OF MOUSE BLOOD

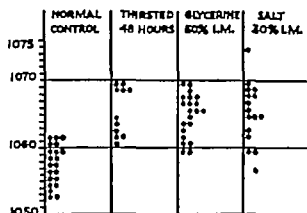


FIG 1

veins before and after administration of glycerine. The red count of untreated mice, given free access to water varied between 8 and 12 million cells. After the administration of glycerine in the majority of instances blood would not flow from the tail vein at all. At first a brachial vein was exposed and incised under ether anesthesia and the freely flowing blood utilized. However, comparative counts on the same animal using blood simultaneously from the tail and from the brachial vein showed poor correlation. Invariably the blood from the tail was more concentrated, ranging from 250,000 to 2 000 000 more red blood cells per c.mm. and having a specific gravity 0.002 to 0.007 greater than blood flowing freely from the brachial vein. Blood from the tail vein was not considered satisfactory and enumeration of red blood cells was not deemed sufficiently accurate in the mouse.

One measure of fluid change within the mouse was the determination of specific gravity of whole blood, by the falling drop technique of Barbour and Hamilton (2). The blood was taken from the brachial vein since tail blood was not a satisfactory index. Multiple successive tests on the same animal before and after the injection of glycerine could not be performed. Consequently whole groups of mice were examined, each mouse serving for a single determination. Results are expressed in Fig 1

In normal mice with free access to water, the range of specific gravity varied from 1 051 to 1 061. In another group receiving 0.35 cc of 50 per cent glycerine, intramuscularly, the blood showed a specific gravity range of 1 059 to 1 069. The examinations were carried out from $\frac{3}{4}$ hour to 3 hours after the injection, at such a time when the animals showed some symptoms of tremor or other systemic disturbance. The slight time variation following inoculation had no bearing on the result.

It is evident that concentration of the circulatory blood volume is a correlate of the action of glycerine. There are other less drastic modes, however, of producing this degree of concentration. If mice are deprived of water for 48 hours, the blood becomes more concentrated and the specific gravity increases to a range of 1060 to 1069, practically identical with the increase produced by

TABLE II
Effect of Thirst and Glycerine in Relation to Intramuscular Virus

Experiment No	Treatment	Dilution of virus suspension	Results
1	Thirsted 48 hrs , no glycerine	10^0	7, 8, 8, 0, 0, 0, 0, 0, 0, 0
	Controls, no thirst or glycerine		3, 3, 4, 0, 0, 0, 0, 0, 0, 0
	Thirsted 24 hrs , plus glycerine		2, 2, 2, 3, 7, X, X, X, X
	Glycerine, but not thirsted		3, 3, 3, 3, 3, 4, 4, 5, 0, X
2	Thirsted 48 hrs , no glycerine	10^{-1}	4, 4, 0, 0, 0, 0, 0, 0, 0, 0
	Controls, no thirst or glycerine		4, 4, 7, 0, 0, 0, 0, 0, 0, 0
	Thirst 48 hrs , no glycerine	10^0	3, 3, 4, 4, 6, 0, 0, 0, 0, 0
	Controls, no thirst or glycerine		3, 4, 4, 4, 5, 0, 0, 0, 0, 0

Animals thirsted for 48 hours received water 14 hours after virus inoculation

glycerine. Such thirsted mice show none of the behavior changes seen after administration of glycerine. When such thirsted animals are inoculated with virus, the rate of death is almost exactly the same as controls, although mice receiving glycerine showed the usual facilitation effect (Table II). In the first experiment it is seen that animals receiving glycerine were much more susceptible to virus than those that did not, regardless of whether the latter were thirsted or not. One group of mice was deprived of water for 24 hours and then given glycerine. In this condition of partial dehydration the glycerine produced a greater initial mortality, but those that survived the initial period succumbed to the virus with greater rapidity. A partial dehydration through deprivation of drinking water renders the facilitation effect from glycerine more striking.

In Experiment 2 of Table II further evidence is presented that thirsted animals not receiving glycerine behave no differently toward the virus than well watered controls.

Evidently the disturbance of which blood concentration is an index must be produced suddenly as by glycerine, since the same degree of hemal concentration produced gradually by thirst is ineffective in facilitating virus action

Dehydration of the Nervous System

Since the degree of hemal concentration may be increased to identical levels by two different procedures, one of which (glycerine) facilitates virus action while the other (thirst) does not, some other differentiating point must be sought. The hypothesis was adopted that changes in the water content of the brain might be of significance

To test this hypothesis the following procedure was adopted

Mice were anesthetized with ether and exsanguinated by section of the brachial arteries and then of the heart. Immediately the brain was removed and weighed in a crucible whose weight had just been determined. The brain was then dried to constant weight in a hot air oven whose temperature ranged from 90° to 100° but did not go above the latter figure. Tissue remained in the oven at least 48 hours. The weight of the dry brain in normal controls as well as in various experimental groups, ranged generally from 0.0800 to 0.0975 gm. depending on the amount of tissue removed from the skull. The wet weight of the normal brain varied from 0.3610 to 0.4463 gm. After administration of glycerine, or as is shown subsequently, of strongly hypertonic sodium chloride the initial (wet) weight of the brain was sharply reduced, to as low as 0.3105 gm. After determination of the dry weight the results were expressed in the ratio of dry to wet brain, that is the percentage of solids

The solids content of the brain of the normal control mice which had been allowed water *ad libitum* averaged 22.1 per cent (Fig. 2). Brains of mice deprived of all water for 48 hours and then treated identically with the controls averaged 22.6 per cent. As seen in Fig. 2, the values are quite closely bunched and the spread not great. The animals into which 50 per cent glycerine was injected intramuscularly, exhibited a much greater spread, with a mean value of 24.8 per cent solids. The great majority of these animals showed typical symptoms of the type described above, and there was a high correlation between the severity of the symptoms, in regard to the tremors and prostration and the degree of cerebral dehydration. Animals with a percentage solids of 26 per cent or greater undoubtedly would have succumbed had they been so allowed. Mice which proved to have a lower percentage of solids (*i.e.*, greater amount of water) would probably have survived.

Tests with Other Substances and Modes of Administration

The above evidence suggests that an acute dehydration of the brain is a differentiating factor which controls the susceptibility to virus. Thirsted mice, in spite of a high concentration of the blood, reacted identically with controls in reaction to the virus. These animals showed only a slight diminution in water content of the brain. Mice receiving glycerine, on the other hand, exhibited a

greater drop in water content, expressed in Fig 2 as the percentage solids. As already demonstrated, these mice had a considerably enhanced susceptibility to virus.

The importance of acute dehydration could be checked in various ways. The possible local action of glycerine on the tissues at the site of inoculation could be eliminated by intravenous administration of glycerine. This is an heroic pro-

PER CENT SOLIDS IN MOUSE BRAIN

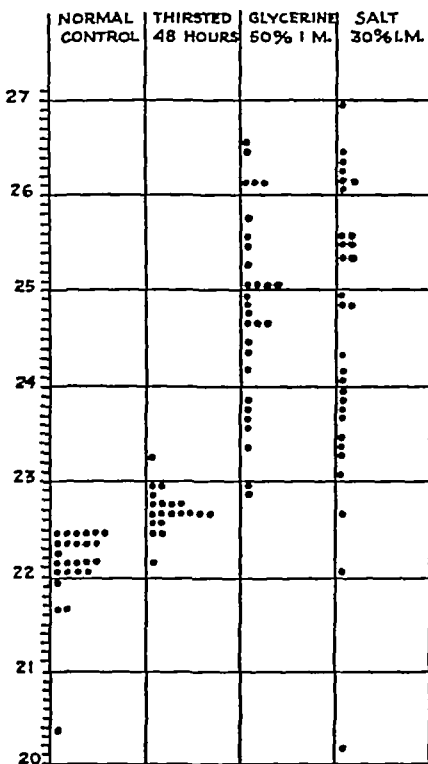


FIG 2

cedure, resulting in immediate death of injected mice in a very high percentage of animals, even with a dose of only 0.15 cc of 50 per cent glycerine. However, in preliminary tests, those that survived the first few hours survived indefinitely. The mice that received glycerine intravenously showed a markedly increased susceptibility to virus given intramuscularly. This is shown in Table III. The treated animals died of encephalitis not only in greater numbers than the controls, but after a shorter incubation period.

Acute dehydration of the tissues can be produced by any strongly hypertonic solution parenterally injected. Experiments were carried out with a 30

per cent solution of sodium chloride, injected in a dose of 0.30 cc intramuscularly. Virus was given simultaneously to these and also to control animals. The injection of the salt caused a high immediate mortality, within a few hours. The survivors however all succumbed to encephalitis. In two experiments, 9 of 9 treated animals died of encephalitis with typical and unmistakable signs, while only 1 of 20 controls died. These data are presented in Table IV. Additional data are given in Table V.

The intramuscular injection of strong salt solution resulted similarly to glycerine in its effect on the specific gravity of the blood and the water content of the brain. In Figs. 1 and 2 are presented the results. The animals treated

TABLE III

Intravenous Administration of Glycerine in Relation to Intramuscular Injections of Virus

Experiment No.	Mice with glycerine	Controls
1	3 3 3 3 3 4 4 4 0	4 0 0 0 0 0 0 0 0
2	2 2 2 3 3 3 4	3 3 4 4 5 0 0 0 0

Animals dying immediately after the glycerine injection are not recorded.

TABLE IV

Intramuscular 30 Per Cent Sodium Chloride in Relation to Intramuscular Virus

Experiment No.	Dilution of virus	With salt	Controls
1	10 ⁰	3 3 4 4	4 0, 0 0 0 0 0 0 0
2	10 ⁻¹	3 3 3 3 4	0 0 0 0 0 0 0 0

Animals dying immediately after the salt injection are not recorded.

with salt showed somewhat greater scatter than those receiving glycerine. Possible reasons for this are commented on in the discussion.

Profound alterations in the electrolyte content of the brain, without significant change of the total water content, may be induced by the injection of distilled water or 5 per cent glucose intraperitoneally. The latter method was extensively employed by Darrow and Yannet (3). There is a shift of electrolytes into the injected fluid, with, for practical purposes, a temporary loss of such electrolytes from the body. Preliminary studies showed that for a 22 gm mouse an inoculation of 40 cc. of either distilled water or of 5 per cent glucose was the maximum amount that could be tolerated intraperitoneally. Animals so treated showed in the course of a few hours some tremors and prostration, although the total picture was not identical with that produced by the injection of glycerine or salt.

Animals subjected to this treatment were inoculated with virus, together with suitable controls. Experiments were run using normal controls, glycerine-treated and salt treated controls. The results are given in Table V. In both

the experiments there shown, only the groups receiving the strongly hypertonic solutions showed significant mortality. Considering the two experiments together, 22 of 27 mice treated with hypertonic solutions succumbed to virus, while of all other groups only 4 of 55 died. Determination of brain water content of mice receiving 5 per cent glucose intraperitoneally showed a strictly normal range, of 20.8 to 22.3 per cent solids, with a mean of 21.9 per cent in 10 determinations.

It is evident that the procedures accompanied by acute dehydration of the brain were correlated with enhanced susceptibility to virus action, while other

TABLE V
Comparison of Glycerine with Other Substances in Relation to Intramuscular Virus

Experiment No	Substance injected	Place	Dose	Results
1	Glycerine (50 per cent)	Intramuscular	0.35 cc	3, 3, 3, 4, 4, 5, 5, 0, 0, 0
	Glucose (5 per cent)	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0, 0
	Distilled water	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0, 0
	None—control	—	—	6, 0, 0, 0, 0, 0, 0, 0, 0, 0
2	Sodium chloride (30 per cent)	Intramuscular	0.30	3, 3, 3, 3, 3, 3, 3, 3, 3, 3
	Glucose (5 per cent)	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	Distilled water	Intraperitoneal	4.0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	None—control	—	—	3, 4, 4, 0, 0, 0, 0, 0, 0, 0, 0

In Experiment 1, undiluted virus suspension was used.

In Experiment 2, 10^{-1} dilution of virus suspension used.

Animals succumbing in a few hours to direct action of injected substances are not included in this table.

procedures wherein the brain was not dehydrated, even though profound electrolyte disturbances were present, showed no increased susceptibility to virus

Other Modes of Virus Administration

The slight invasiveness, under normal conditions, of equine encephalomyelitis virus, does not apply to intranasal or intraocular injections. The effectiveness of these modes of inoculation has been previously described and discussed (1a). The problem then arose, would the facilitation effect produced by glycerine apply to cases where virus could normally attain the nervous system quite readily?

Experiments were carried out, wherein virus suspensions in different dilutions were given into the eye or nose of control mice and of those receiving intramuscular glycerine, (0.35 cc of 50 per cent solution). Since the controls would

ordinarily succumb, any facilitation effect would be evidenced principally in a shortened incubation period, as well as possibly a higher titration end point.

The results are seen in Table VI. The animals receiving glycerine appear almost identical with the controls. The facilitation effect is produced by glycerine when the virus is injected intramuscularly, but in cases of intranasal or intraocular injections no facilitating action can be detected.

TABLE VI

Intramuscular Glycerine in Relation to Intraocular and Intranasal Administration of Virus

Virus injected	Dilution	With glycerine	Control
Intraocularly	10^{-4}	3 4 4 5 5 0 0	4 4 4 5 6 0 0 0
	10^{-6}	3 3 3 4 4 6 0	3 3 4 4 5 5 5 0
	10^{-2}	3 3 3 4 4 4 4	3 3 3 4 4 4 4 \
	10^{-1}	3 3 4 4 4 4 4	3 3 4 4 4 4 4 4
	10^0	N T	N T
Intranasally	10^{-4}	0 0 0 0 \ \ \ \ \	0 0 0 0 0 0 0 0
	10^{-6}	4 7 0 0 0 0 0 0	0 0 0 0 0 0 0 0
	10^{-2}	3 4 4 4 4 0 0 \	3 3 3 3 5 5 0 0
	10^{-1}	3 3 4 0 \ \ \ \	3 3 4 4 4 4 0 \
	10^0	N T	N T

DISCUSSION

The action of glycerine on the animal organism has been exhaustively reviewed by Deichman (4). The minimal lethal dose for mice has been worked out by Leake and Corbitt (5), whose findings are close to our own. They found that animals surviving 24 hours would survive indefinitely, which we have confirmed. No data in the literature really bear upon the cause of death from too much glycerine. The present studies indicate that excessive loss of water from the brain is a responsible factor. Animals with the most pronounced symptoms showed the most severe dehydration. A preliminary period of thirst (18 to 24 hours) rendered animals much more susceptible to glycerine and incidentally, to the virus action following glycerine administration.

Hemoglobinuria is produced in mice by glycerine, but is not a causative factor in the phenomenon under investigation. The use of salt solution causes the same facilitation phenomenon with the same degree of brain dehydration, but does not result in hemoglobinuria.

The use of 30 per cent sodium chloride renders most of the mice intensely thirsty. If water is not furnished until 3 to 4 hours after the injection, about 80 per cent of the animals die. Curiously enough, with water available, some of the animals show no inclination to drink while some consume very large amounts. It is the latter which furnish the very low values in Fig. 2, and show no symptoms of shock or prostration.

Animals receiving glycerine exhibited far less thirst than the salt group. This finding is in agreement with the studies of Gilman (6) on the relative actions of sodium chloride and of urea.

The way in which the virus, intramuscularly injected, attains the nervous system, is not considered in the present paper. Sabin and Olitsky (7) have presented evidence for the mouse, while the author has considered the mechanism in the guinea pig (8). Just how the dehydration of the brain allows the virus to enter, whereas otherwise it would not, is not at present clear. The absence of facilitation when virus is instilled into the nose or injected into the eye, suggests a fundamentally different mechanism for these routes than for the intramuscular. These results are in complete harmony with the author's previous studies on herpes and pseudorabies viruses (1 b), and indicate a sharp difference between direct intraneural route of invasion and mediation of infection through the blood stream. The effectiveness of glycerine evidently applies only to the latter category.

SUMMARY

50 per cent glycerine injected intraperitoneally, intramuscularly, or intravenously, greatly enhances the activity of equine encephalomyelitis virus injected intramuscularly, increasing its virulence up to 100-fold. The same effect is produced by very concentrated sodium chloride. The result appears due to dehydration of the nervous system, suddenly produced. Gradual withdrawal of body fluids, produced by depriving animals of drinking water, results in sharp concentration of the blood, equal to that produced by glycerine or salt. But such deprivation of water alone does not result in significant dehydration of the brain, nor does it have any effect on virus action. The facilitation effect is not produced by drastic procedures involving shifts of electrolytes without loss of total water from the brain. Glycerine has no facilitating action when the virus is administered intranasally or intraocularly, suggesting a fundamental difference in pathogenesis between these routes and the intramuscular.

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THE FORMATION OF ANTIBODIES IN THE POPLITEAL LYMPH NODE IN RABBITS*

BY W. E. EHRLICH, M.D., AND T. N. HARRIS, M.D.

(From the Departments of Pathology, Bacteriology, and Pediatrics The School of Medicine University of Pennsylvania, Philadelphia)

PLATES 26 AND 27

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Among the authors who have supported the view that antibodies are formed in lymph nodes McMaster and Hudack (1) seem to be the only ones who have presented evidence which may be regarded as conclusive. These authors injected 0.02 cc. of solutions of various antigens intradermally into the ear of mice, then excised the regional cervical lymph nodes, ground those of 20 to 30 mice with sand, and determined the agglutinin titer in the extract. By this method McMaster and Hudack were able to detect agglutinins in the lymph nodes about 1 week after the injections, and in the beginning they found a slightly higher titer in the lymph nodes than in the serum or the tissue of the ear. Excision of the ear 3 hours after injection did not interfere with agglutinin formation. Furthermore, fixation in the injected tissue as described by Menkin (2) could be ruled out by injecting different antigens into the two ears. Accordingly McMaster and Hudack concluded that agglutinins are formed within the draining lymph nodes in mice.

The cells which form antibodies have not yet been definitely identified, although some hold that cells of the reticuloendothelial system are involved. Sabin (3) suggests that it is the monocyte, the globulin of its cytoplasm being transformed by the action of the antigen into antibody globulin. However, convincing evidence of this has never been presented. On the contrary, there are observations which are not in agreement with this concept. It has been observed, for instance, that after large doses of antigen which stimulated proliferation of reticuloendothelial elements in various organs the antibody titer remained low, whereas with small doses which did not produce visible proliferation of these cells, high titers were obtained (4, 5). Moreover, following intravenous injection of staphylococcus vaccine in experimental animals the rise in antibody titer in the serum has been found to parallel the activity of the Malpighian bodies of the spleen (5). Finally Rich, Lewis, and Wintrobe (6) concluded that the large mononuclear cells in the spleen which responded so specifically to parenteral injection of foreign protein as to be suspected of rela-

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tionship to the process of antibody formation were not histiocytes, but resembled lymphoblasts in all observed respects. Although these cells were not necessarily lymphoblasts in the sense of being immature lymphoid cells, it was conceivable that they were lymphocytes which were greatly enlarged because they performed some special function.

In order to arrive at a better understanding of these questions, it seemed desirable to gather more precise knowledge of the events that take place in the lymph node during antibody formation. We therefore compared the production of antibodies and cells in the lymph node, and analyzed the simultaneous morphologic changes in the lymphatic tissue. It was found that the popliteal lymph node of the rabbit was particularly suitable for our investigation because it is the sole node that drains the hind foot, and is large enough to allow a separate study of all parts of the system: the local tissue injected with the antigen, the afferent lymph, the popliteal lymph node, the efferent lymph, and finally the serum.

Material and Method

The rabbits injected were male Chinchillas weighing about 2000 gm. The antigens used were typhoid bacilli and sheep erythrocytes, and the antibodies determined were typhoid-agglutinin, erythrocyte-hemolysin, and erythrocyte-agglutinin. For control studies in 16 animals the opposite leg was injected with 1 per cent solution of crystalline egg albumin while in 9 others the opposite leg was not injected at all (Table II).

The antigens were prepared as follows. The typhoid bacilli were taken from an 18-hour culture of an old laboratory strain O 901, washed 3 times by centrifugation in saline, heated in the 56°C water bath for 1 hour, and then suspended in an approximately 50 per cent suspension. The sheep erythrocytes were collected from blood drawn within the week, washed 3 times, and suspended in approximately 50 per cent suspension. In all cases 0.2 cc. of antigen was given subcutaneously in the plantar surface of the hind foot, close to the toes, and the puncture wound was closed with collodion. Details of the individual experiments are given in Tables I and II.

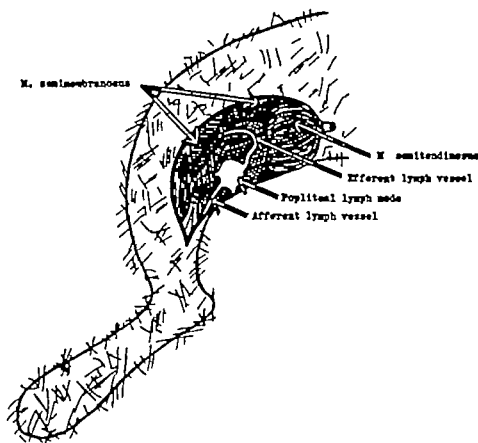
For the collection of the lymph the animal was anesthetized with amytal, the skin of the inner aspect of the knee incised, the semitendinosus and semimembranosus muscles cut, and the popliteal lymph node exposed (Text-fig 1). The lymph was collected through a 27-gauge needle into a "tuberculin" syringe moistened with sodium citrate solution. A ligature placed around the lymphatic served to distend it and provided a better hold on the vessel. By this method we were able to collect from the efferent vessel an average of 0.17 cc. of lymph within 7 minutes, and occasionally as much as 0.6 cc. in 6 minutes (Tables I and II). After the lymph had been obtained the animal was killed by ether. Then blood was collected from the heart, the lymph node was excised, weighed, and fixed in Helly's fluid, and finally the sections were stained with Azur II-eosin.

The determination of antibodies presented a technical problem because of the

small yields of lymph. Occasionally the amounts collected were too small for use. The dilutions were in 0.1 cc. quantities, usually in steps of two, done as far as possible with Kahn pipettes. For the respective tests the technique was as follows —

1 The typhoid bacillus agglutination was done by incubating equal quantities of the dilution of lymph or serum and antigen suspension overnight at 50°C. The antigen was prepared from the suspension used for injection, and was quite light.

2 Two tests were performed in the cases of the erythrocytes. The hemolysin test was done by mixing 0.1 cc. quantities each of diluted serum or lymph, 5 per cent



TEXT FIG. 1. Diagram showing the location of the popliteal lymph node and its lymph vessels in the right leg of a rabbit.

sheep erythrocyte suspension, and 1/10 pooled guinea pig complement. The mixture was made up to 0.5 cc. with saline and incubated for 30 minutes. The agglutination tests were done by adding 0.1 cc. quantities of serum or lymph dilution to like quantities of a 1 per cent erythrocyte suspension. All rabbits were tested before injection for sheep erythrocyte antibodies.

Lymph node extract was prepared by drying the lymph node in accordance with the Cryochem¹ process then extracting it in a mass of water equal to the weight of tissue dried. Tissue extract from the site of injection was similarly prepared.

¹The Cryochem process consists of dehydration of solutions from the frozen state the freezing being accomplished by reduction in atmospheric pressure (Flösdorff, E. W., and Mudd, S. *J. Immunol.*, 1938, 34, 469).

RESULTS

The Time and Site of Appearance of Antibodies

In preliminary experiments a comparative study was made of the antibody titers at the site of injection, in the afferent lymph, in the substance of the

TABLE I

Site and Time of Antibody and Lymphocyte Formation Following Subcutaneous Injection of Typhoid Vaccine into the Left Foot, and Sheep Erythrocytes into the Right

Left foot(injected with typhoid vaccine)									
Rabbit No	Duration of experiment	Agglutinin titer vs <i>E typhosa</i>					No of lymphocytes per c mm of lymph		
		Foot tissue	Afferent lymph	Lymph node	Efferent lymph	Blood serum	Afferent lymph	Efferent lymph	
	days								
102	3	0	0	4	4	16	14,000	78,900	
43	4	0	2	128	32	64	4,300	41,600	
44	5	16	—	512	64	256	10,000	46,000	
57	5	—	16	128	192	256	3,150	144,000	
45	6	16	16	1024	64	512	2,700	80,600	
58	6	—	12	256	256	256	6,000	82,000	
61	9	0	12	128	64	768	7,600	43,800	

Right foot (injected with sheep erythrocytes)										
Rabbit No	Agglutinin titer vs erythrocyte				Hemolysin titer vs erythrocyte				No of lymphocytes per c mm of lymph	
	Afferent lymph	Lymph node	Efferent lymph	Blood serum	Afferent lymph	Lymph node	Efferent lymph	Blood serum	Afferent lymph	Efferent lymph
102	0	0	0	0	0	0	0	0	3,700	73,600
43	0	0	0	0	0	48	16	12	2,100	41,200
44	0	32	16	48	8	2048	256	128	5,000	28,050
57	—	32	32	64	—	64	128	64	2,000	55,400
45	0	192	32	64	12	1024	768	512	3,200	72,100
58	—	32	64	64	—	128	192	512	5,750	97,700
61	12	16	16	48	32	64	48	256	1,100	54,600

lymph node, in the efferent lymph, and in the serum. Seven rabbits were injected with typhoid vaccine in the left hind foot and sheep erythrocytes in the right. The results of these experiments are presented in Table I. It can be seen that in the area of injection and in the afferent lymph the titers remained very low. Moreover, one notes that a great increase in titer took place within the lymph node, and that in some cases titers of lymph node extract and of efferent lymph were higher than those in the serum at the same time. This was found in some typhoid and some erythrocyte experiments. It is also apparent

that the number of leucocytes per c.mm. were grouped about an average of 5000 in the afferent lymph, and about 67,000 in the efferent vessel. Inasmuch as a comparable protein concentration in afferent and efferent lymph excludes absorption of water in the lymph node (Drinker and Yoffey (7)), our results can be taken to mean that in the rabbit antibodies are formed within the draining lymph node. Furthermore, this conclusion is valid for different types of antibody reaction.

The Relative Antibody Titer in Lymph and Serum

Let us now turn to our main series of experiments and compare the antibody titers in the efferent lymph of the injected foot with the titers found in the serum and in the lymph of the other foot. It can be observed from Table II that the titers in the serum at first lagged slightly behind those in the efferent lymph of the injected foot, but soon rose to figures that were considerably higher, the peaks being reached on or about the 9th day, that is, after the peaks in the lymph had been passed. The titers in the efferent lymph of the opposite foot remained very low throughout the experiment. As a matter of fact, they never reached figures greater than would be expected as a result of leakage of antibodies into the tissues from the circulating blood.

The Relation of Antibody Titer of Lymph to the Cells of the Lymph and to the Weight of the Lymph Node

In comparing the antibody titers in the efferent lymph of the injected foot with the number of cells in this lymph and the weight of the lymph node, we find (Table II and Text fig. 2) that in the typhoid experiments agglutinins appeared in the efferent lymph as early as the 2nd day, however the titer began to rise steeply only the 4th day. They reached an average of about 1:190 on the 6th day and thereafter declined slowly. The number of cells rose from an average of 17,000 per c.mm. before injection (12 control experiments) to an average between 50,000 and 60,000 on the 3rd day and maintained this level for at least 3 weeks. The weight of the lymph node rose from an average of 0.19 gm. to one of almost 1.00 gm., the latter being observed on the 9th day.

In the erythrocyte experiments similar observations were made. Here too the titers did not begin to rise steeply until the 4th day, although hemolysins had appeared on the 3rd day, and the agglutinin titer rose to an average of about 1:32, and the hemolysin titer to 1:230 both on the 6th day. However, in the erythrocyte experiments the titers fell more quickly than in the typhoid experiments, the agglutinins having disappeared from the lymph as early as 14 days after injection, and the hemolysins after 29 days. The number of cells and the weight of the lymph node showed similar changes. The number of cells rose to an average between 50,000 and 60,000 per c.mm., which lasted less than a fortnight and the average weight of the lymph node did not rise higher than

TABLE II

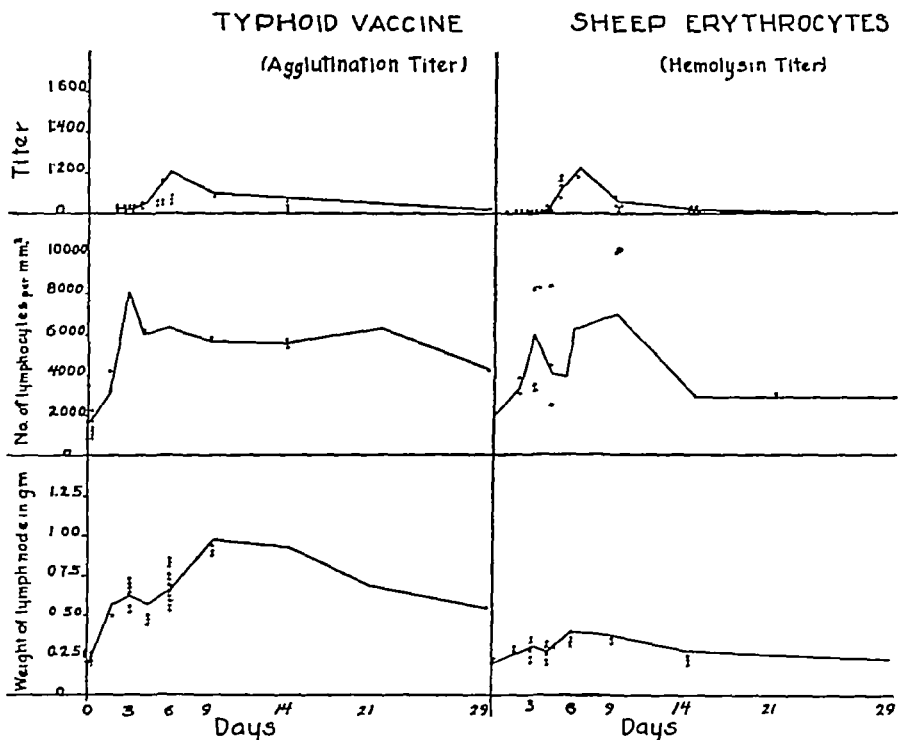
LEFT										RIGHT									
Weight of rabbit	Antigen used	Effer cent lymph col lected	Time of col lection	No of lympho cytes per c mm of lymph	Agglutination iter			Weight of lymph node gm	Effer cent lymph col lected	Time of col lection min	No of lympho cytes per c mm of lymph	Agglutination iter			Hemolysis iter			Weight of lymph node gm	
					Same side	Oppo site side	Se rum					Same side	Oppo site side	Se rum	Same side	Oppo site side	Se rum		
1-3	No	0.04	6	18 000				0.2	No	0.05	7	20 700						0.2	
1-4	"	0.11	6	16 800				0.2	"	0.11	5	17 300						0.2	
2-4	Typhoid	—	—	—	—	0	0	0.35	Erythrocytes	0.02	6	56 800	0	—	0	0	—	0.2	
1-1	"	0.05	6	31 600	2	0	0	0.4	"	0.11	7	32 000	0	0	0	—	—	0.3	
2-0	"	0.17	10	23 200	2	0	0	0.75	"	0.02	—	22 500	0	0	0	0	—	0.25	
7-3	"	—	—	—	—	—	—	0.65	Egg	0.39	6	17 900	0	—	0	—	—	0.2	
1-9*	"	0.02	—	40 500	—	—	—	0.5	No	0.14	6	22 000	0	0	0	0	—	0.2	
3-8*	Egg	0.055	6	60 400	0	0	0	0.2	Erythrocytes	0.13	6	41 200	0	0	0	0	—	0.2	
2-1	No	0.04	6	13 600	—	—	—	0.15	"	0.03	5	33 600	—	—	—	—	—	0.2	
2	Typhoid	0.12	15	26 800	4	—	16	0.5	Typhoid	—	—	—	—	4	16	—	—	0.7	
6	"	0.01	10	204 000	4	0	16	0.7	Erythrocytes	0.03	13	32 000	0	0	0	0	0	0.2	
1-2	"	—	—	—	—	—	2	0.5	"	0.02	11	34 100	0	—	0	0	—	0.3	
1-5	"	—	—	—	—	—	8	0.7	"	0.09	8	49 800	0	—	0	0	—	0.3	
3-4	"	0.19	4	44 900	2	0	8	0.65	"	0.03	4	82 400	0	0	0	0	—	0.25	
1-02	"	0.445	6	78 900	4	—	16	0.55	"	0.13	6	73 600	0	—	0	0	—	0.325	
9-7	"	0.03	2	172 800	0	0	4	0.85	Egg	0.03	2	68 000	0	0	0	0	—	0.25	
3-9*	Egg	0.10	6	122 800	0	0	0	0.4	Erythrocytes	0.13	6	87 400	0	0	0	0	—	0.45	
7	Typhoid	0.26	15	59 300	256	64	128	0.7	"	0.11	15	14 800	0	0	0	0	0	0.2	
1-6	"	0.09	5	133 300	48	4	16	0.5	"	0.03	5	44 000	0	0	0	0	—	0.3	
3-3	"	0.17	5	59 600	64	16	128	0.5	"	0.025	4	84 800	0	0	0	0	—	0.25	
4-3	"	0.13	5	41 600	32	—	64	0.75	"	0.13	5	41 200	0	—	0	0	—	0.375	
2-2	"	0.46	6	13 000	16	4	64	0.45	Egg	0.08	5	20 000	0	0	0	0	—	0.15	
3-3*	"	0.46	6	21 400	12	2	64	0.5	No	0.11	6	8 700	—	—	—	—	—	0.15	
9-4*	Egg	—	—	—	—	—	0	0.25	Erythrocytes	0.02	6	22 400	—	—	—	—	—	0.15	
2-3	No	0.08	6	17 000	—	—	—	0.15	"	0.10	8	34 800	0	0	0	0	—	0.25	
5-3	Typhoid	0.215	6	98 400	256	—	—	0.5	Typhoid	0.105	6	90 000	256	—	—	—	—	0.6	
5-4	"	0.70	6	14 250	64	—	—	0.35	"	0.17	6	20 400	128	—	—	—	—	0.3	
4	"	0.03	8	19 700	128	16	256	0.8	Erythrocytes	0.07	15	27 600	16	0	64	128	8	0.45	
1-7	"	0.13	3	86 000	64	16	192	0.9	"	0.08	3	55 000	12	0	48	128	16	0.35	
3-6	"	0.42	6	35 000	192	64	256	0.85	"	0.06	5	59 600	12	0	64	192	8	0.3	
4-4	"	0.365	6	46 000	64	—	—	0.65	"	—	—	—	—	—	—	—	—	—	

6	3	2110	Typhoid	0.05	6	91 600	64	—	192	0.6	Typhoid	0.11	20	125 100	64	—	192	48	256	48	1024	0.7
5-5	2005	2110	"	0.37	6	26 200	512	—	—	0.65	"	0.48	6	21 400	1024	—	—	—	—	—	0.6	
5-4	2210	2110	"	0.85	6	33 600	512	—	—	0.85	"	0.68	6	39 500	256	—	—	—	—	—	0.75	
5	2180	2110	"	0.11	15	54 200	1024	128	2048	0.8	Erythrocytes	0.01	13	576 000	256	48	192	256	48	1024	0.5	
1-8	1620	—	—	—	—	—	—	—	1024	0.7	"	—	—	—	—	—	—	—	—	—	0.4	
3-7	1360	58 500	192	0.22	6	80 600	64	64	768	0.55	"	0.265	6	65 000	64	2	16	512	16	256	0.3	
4-5	1940	80 600	64	0.45	6	82 000	256	—	512	0.7	"	0.16	6	72 100	32	—	64	768	—	512	0.375	
5-8	1925	82 000	256	0.05	6	81 900	128	48	256	0.7	"	0.27	6	97 700	64	—	64	192	—	512	0.5	
8-7	1810	81 900	128	0.27	6	47 000	12	—	256	1.1	Egg	0.14	6	19 200	0	0	0	—	—	—	0.35	
2-2	2040	47 000	12	0.24	8	19 000	0	—	32	1.25	No	—	—	—	—	—	—	—	—	—	0.25	
7-4	1900	19 000	0	0.27	6	13 400	0	0	3	0.2	Erythrocytes	0.195	6	30 600	16	0	32	128	4	256	0.3	
2-8	1990	13 400	0	0.20	6	—	—	—	—	0.15	"	0.21	6	51 100	16	0	48	64	2	512	0.4	
9	8	2000	Typhoid	0.05	9	66 200	256	8	1024	0.9	"	0.02	13	21 000	<16	16	256	<16	512	3072	0.5	
2-6	2110	64	64	0.13	5	47 200	64	64	512	1.05	"	0.095	5	100 400	8	16	32	48	64	1024	0.4	
5-9	2090	—	—	—	—	—	—	—	—	0.9	"	0.01	6	134 500	8	—	12	64	—	768	0.35	
6-1	1940	43 800	64	0.38	6	58 300	64	16	768	0.9	"	0.09	6	54 600	16	—	48	48	—	256	0.3	
9-8	1710	58 300	64	0.22	6	12 300	0	0	256	1.05	Egg	0.07	6	29 600	0	0	0	—	—	—	0.2	
4-0	1680	12 300	0	0.205	6	—	—	0	0	0.2	Erythrocytes	0.14	1	32 200	8	0	16	32	2	256	0.3	
2-7	2060	35 200	0	0.03	3	—	—	—	—	0.25	"	0.04	5	78 200	12	<8	16	64	6	512	0.45	
14	9	2140	Typhoid	0.17	15	53 100	32	16	1024	0.85	"	0.12	9	45 900	0	0	32	64	64	256	0.25	
2-5	1860	70 000	16	0.145	10	21 300	64	64	256	0.85	"	0.14	6	21 700	0	0	0	16	16	64	0.35	
6-0	2110	21 300	64	0.41	6	61 600	32	8	192	0.4	"	0.11	6	35 100	0	0	0	32	8	512	0.25	
9-9	1760	61 600	32	0.115	6	46 700	1024	16	128	1.45	Egg	0.14	6	14 000	0	0	0	—	—	—	0.35	
2-9	1800	46 700	1024	0.26	5	8 200	0	0	256	1.0	No	0.07	5	10 000	0	0	0	16	8	32	0.2	
4-1	1820	8 200	0	0.175	6	—	—	0	0	0.15	Erythrocytes	0.21	6	15 000	0	0	0	48	8	256	0.25	
2-3	1880	9 600	0	0.03	3	—	—	—	—	0.15	"	0.205	6	16 500	0	0	0	—	—	—	0.25	
21	2060	29 800	64	0.205	5	86 200	32	12	256	0.6	"	0.10	7	21 000	0	0	0	4	16	64	0.2	
1-00	1650	86 200	32	0.145	6	12 800	0	0	64	0.15	Egg	0.07	6	45 900	0	0	0	—	—	—	0.25	
4-2	1940	12 800	0	0.165	6	—	—	0	0	0.2	Erythrocytes	0.165	6	37 200	0	0	0	4	2	8	0.3	
29	1-01	2080	Typhoid	0.17	6	41 650	8	0	12	0.55	"	0.21	6	21 600	0	0	0	0	0	16	0.3	

Showing the antigen used, the amount of effluent lymph collected, the number of lymphocytes in the effluent lymph, the antibody titers in the effluent lymph of the same node and of the opposite node as well as in the serum, and the weight of the popliteal lymph node, in the left and in the right leg of 64 rabbits.

* In these animals the left has been exchanged for the right.

0.4 gm on the 6th day. It should be noted that in both the typhoid and erythrocyte experiments, the rise in antibody titer was preceded by the rise in cell number and weight of lymph node.



TEXT-FIG 2 Showing the antibody titers and number of lymphocytes in the efferent lymph as compared with the weight of the lymph nodes following the injection of typhoid vaccine into the left foot, and sheep erythrocytes into the right

The Nature of the Cells in the Lymph

In making films for differential counts the same difficulties were encountered which Rous (8) and Drinker and Yoffey (7) described and attributed to the lack of "body" or protein content of the lymph as compared with blood. Nevertheless the film method was used, because approximate values seemed to be all that was needed. In 14 typhoid experiments in which this method was employed the percentage of small lymphocytes was found to drop from 98 or 99 per cent before injection to an average of 93 per cent during the 1st week of the experiment, while the percentage of large lymphocytes rose from 2 or 3 per cent to an average of 6 per cent, the largest single observation being to 11 per cent. Monocytes were scarce except during the first 3 or 4 days when some rabbits

showed 1 or 2 per cent of these cells, while the granulocytes in some rabbits amounted to 4 per cent on the 2nd day, and to 2 per cent on the 3rd. Similar results were obtained in 9 erythrocyte experiments though the changes were less marked.

Morphologic Changes in the Lymph Node

The morphologic changes observed in the regional lymph node were essentially the same in both series of experiments, although in the typhoid series they were more severe than in the others. In all cases the changes began with a granulocytic infiltration and the appearance of some monocytes. This was followed by a rather diffuse lymphoid hyperplasia, with or without solid secondary nodules, and on the 4th day was superseded by an appearance and enormous hyperplasia of Flemming's secondary nodules (so called germinal centers) which lasted until the end of the experiments.

In the typhoid series, large numbers of granulocytes were found in the marginal sinus after 3 hours, after 6 hours they were seen also in the interior sinus as well as in the postcapillary veins and their neighborhood, and a few hours later they had infiltrated some of the secondary nodules. After 1 and 2 days numerous granulocytes both free and within macrophages, were seen everywhere in the sinus and in the secondary nodules. On the 3rd and 4th days many granulocytes had been phagocytosed, and on the 5th day they had practically disappeared.

The granulocytic infiltration was soon followed by the appearance of typical monocytes, of the size of large lymphocytes, with bean-shaped nuclei and pale cytoplasm. These cells were first seen in the marginal sinus after 16 hours (Fig 7), and in a day or two many were found also in and near the postcapillary veins. The sinus continued to contain monocytes after the granulocytes had disappeared, though their number progressively decreased.

In the lymphatic tissue proper we first observed enlargement of the preexisting secondary nodules, both the solid nodules and those of Flemming's type. A zone of large gray blue lymphoid cells appeared along the marginal sinus, particularly around the secondary nodules, occasionally even replacing them (Fig 8). This area closely resembled the outer zone so often found around the Malpighian bodies of the spleen. On the day following the injection a few secondary nodules of Flemming's type were still present, but after 2 days they had disappeared in the cortex. The latter was now tremendously enlarged and consisted of a diffuse lymphoid tissue (Figs 3 and 4) which contained many large lymphocytes and mitotic figures. This picture continued until the 4th day. Small or indistinct secondary nodules of Flemming's type then made their appearance, and became prominent on the 6th day. After 9 and 14 days there were numerous large confluent Flemming's secondary nodules (Figs 5 and 6) the diameter of the pale centers being 3 to 4 times that of the centers in

our controls (Figs 1 and 2) There were many mitoses in the pale centers, and numerous tingible bodies were present in the macrophages The secondary nodules began to decline after 21 days, although they continued to be prominent until the end of the experiment

In addition to these changes, small foci of epithelioid cells were found in the sinus and lymphatic tissue in 10, and large foci in 4 of 18 animals which were sacrificed after 6 days or later (Figs 10 and 11) Extramedullary myelopoiesis (granulocytopoiesis) was found in the centers of some Flemming's secondary nodules in 9 animals of 18 which were killed after 6 days or more

Our erythrocyte experiments resembled the typhoid experiments in that many granulocytes were found in the marginal and interior sinus as well as in and around the postcapillary veins on the day following the injection However, after 2 days only occasional granulocytes were found, and after 4 days they had disappeared

Monocytes were conspicuous in the sinus only during the 1st day after injection, although a few were to be found for 2 or 3 days thereafter In the animal which was sacrificed after 1 day (rabbit 2-4), large numbers of monocytes were seen in an efferent lymph vessel as well (Fig 9) This seems to indicate that these monocytes had arrived from the periphery of the system

The preexisting secondary and pseudosecondary nodules also underwent swelling After 3 days, however, although distinct pseudosecondary nodules were still visible, a more diffuse hyperplasia resembling that in the typhoid experiments was generally in evidence Flemming's secondary nodules did not entirely disappear except in 1 of 4 animals sacrificed on the 2nd day, and in 1 of 5 animals sacrificed on the 3rd day On the latter day many large lymphocytes were found in the lymphatic tissue, and mitoses were frequent As in the typhoid experiments, Flemming's secondary nodules became larger and more distinct on the 4th day Their diameter reached 2 or 3 times the normal size on the 9th day, and thereafter they became progressively smaller There were many mitoses in the pale centers, and numerous tingible bodies were present in their macrophages

The large nests of epithelioid cells observed in some typhoid experiments were conspicuously absent in the erythrocyte series, although small foci were found in 8 of 17 animals sacrificed after 6 days or more, whereas extramedullary myelopoiesis in Flemming's secondary nodules was observed in 11 rabbits of 32 which were killed after 3 days or later (In 8 of the latter the other foot had been injected with typhoid vaccine, and in 3 with egg albumen)

Finally, in the egg albumen experiments, only a very few granulocytes and monocytes were seen in the popliteal lymph node 2 and 3 days after injection, but the hyperplasia of the lymphatic tissue was almost as marked as in the erythrocyte experiments As a matter of fact, in the two animals which were sacrificed after 3 days the cortex was diffusely enlarged and consisted of large pseudosecondary nodules with only solid secondary nodules, and Flemming's

secondary nodules appeared in 4 days and reached their peak on the 6th day, when their diameter had about doubled. Small or moderately large nests of epithelioid cells were seen in the lymphatic tissue in the 4 animals sacrificed after 14 and 21 days, but extramedullary myelopoiesis in Flemming's secondary nodules was not observed.

DISCUSSION

Comparison of Our Data with Those in the Literature—Before attempting to correlate our data let us briefly consider whether or not they should be regarded as representative. Comparative data on the number of cells in the lymph of peripheral lymph nodes in rabbits are scanty, except in the Japanese literature, which is not readily available. Under normal conditions, the afferent lymph of the popliteal lymph node has been found to contain from 1100 to 3500, or averages of from 2050 to 2200 white cells per c.mm. (Nii (9), Okaue and Hojo (10)), while the efferent lymph of this node contained from 1000 to 20,400, or averages of from 5200 to 9900 cells (Nii (9), Nakagawa (11), Horu (12)), and that of axillary lymph nodes from 9000 to 24,000, or an average of 14 650 cells (Menkin and Freund (13)), whereas after subcutaneous injection into the foot of *lycopodium*, egg albumen, frog blood, etc., the number of white cells in the efferent lymph of the popliteal lymph node rose to averages of 22,000 to 50 000 per c.mm. (Horu (12)). Our own data are in good accord with the above figures, the number of white cells in the efferent lymph of our normal rabbits, for instance, amounting to an average of 17,000 cells, which is especially close to the data of Menkin and Freund.

As to the differential counts of the white cells in the efferent lymph of normal rabbits, we found from 99 to 100 per cent lymphocytes and from 0 to 1 per cent monocytes, which agrees with the data of Menkin and Freund (13), Nii (9), Aoki (14), and Nakagawa (11). However, we observed a larger number of large lymphocytes and a smaller number of small lymphocytes in the efferent lymph. Finally our finding relatively few monocytes and granulocytes in the efferent lymph in comparison with the afferent lymph and in the lymph node proper is of particular interest since Drinker and Yoffey observed (7) an unusually high percentage of monocytes in the peripheral lymph as compared with values reported for the central lymph. These observations can mean only that in our experiments these granulocytes and monocytes were retained or filtered off in the lymph node.

Our antibody titers compare well with those of McMaster and Hudack (1). As to the time of their appearance our method allowed a somewhat earlier detection than that reported by these authors.

The Correlation and Interpretation of the Data—First in the chain of events following the injection was the appearance in the sinus of granulocytes and monocytes, some of which arrived by way of the lymph stream, while others seemed to emigrate from the postcapillary veins within the lymphatic tissue. The latter observation seems to indicate that part of the antigen had been

carried through the lymph into the lymph node, where it caused exudation. The appearance of granulocytes and monocytes was soon followed by general enlargement of the lymphatic tissue, including the preexisting secondary nodules. This enlargement was due chiefly to the appearance of small and medium sized lymphocytes, although increasing numbers of large lymphocytes were also observed. The enlargement of lymphatic tissue was accompanied by an increase in weight of the lymph node and by the output of large numbers of cells, especially small lymphocytes, through the efferent lymph, all these reactions reached their peak 3 days after injection of the antigen, this peak being apparent in all three experiments.

The second phase, appearing on the 4th day and reaching its peak on the 6th to the 9th day, was characterized by the appearance of antibodies in the efferent lymph and serum and by the development of large secondary nodules of Flemming's type (so called "germinal centers") in the lymph node. The weight of the lymph node continued to increase, and the number of lymphocytes in the efferent lymph remained elevated. Thus the rise in antibody titer in the efferent lymph followed on the heels of the increase in the number of lymphocytes in this lymph, but coincided or preceded the development of Flemming's secondary nodules in the lymph node.

In interpreting these results we shall first see whether they point to the so called germinal centers as the site of production of antibodies, and then whether they point to the lymphocytes or the reticuloendothelial cells as the cells concerned.

It is true that the secondary nodules erupted or developed simultaneously with the antibodies. However, in the typhoid experiments at least, they reached the peak of their development only after the peak of antibody formation had passed, and they continued to be active long after the antibody titer had begun to decline.

As to the reticuloendothelial cells, we observed that they became active during the experiments, and that in some cases large foci of epithelioid cells were observed. However, marked proliferation of epithelioid cells was seen only in a few typhoid experiments, and it would seem that this was due to the same probably toxic factors which cause the well known reticuloendothelial reaction in human typhoid fever. On the whole, the reticuloendothelial cells remained quite inconspicuous, foci of epithelioid cells appeared only where granulocytes were engulfed, they were in no way parallel to the antibody titers.

On the other hand, the lymphocytes increased enormously in number and there was a great rise in lymphocyte output through the efferent lymph. These events took place shortly before the antibodies made their appearance. We feel that this observation alone suggests that the lymphocyte may play an important rôle in antibody formation.

We have no information at present on the nature of the rôle which the lymphocyte may play in antibody formation. It may be that it is not concerned in

the production of antibodies, but has some other function of which we are not yet aware. On the other hand, it may take an active part in the elaboration of antibodies. It is true that it does not phagocytose and therefore cannot absorb corpuscular matter. But it may have the faculty of absorbing dissolved antigens or their split products, and it may perhaps go into action only after the raw material has been properly prepared by the action of phagocytes or other mechanisms. However this may be, our results lend little support to the idea that the antibodies are products of the reticuloendothelial cells alone. This latter concept, in fact, is hardly consistent with the complex chain of events in the lymph node during the formation of antibodies as we have here described it.

SUMMARY

Typhoid vaccine and sheep erythrocytes were injected subcutaneously into the feet of rabbits, and the subsequent formation of agglutinins and hemolysins in the popliteal lymph node was compared with the output of lymphocytes through the efferent lymph and with changes in the lymph node.

Antibodies began to appear in the efferent lymph 2 to 4 days after the injection of the antigen and reached their highest titer after 6 days. This was preceded by a sharp rise in the output of lymphocytes through the efferent lymph, while in the lymph node there was lymphatic hyperplasia after preliminary infiltration of granulocytes and monocytes. This hyperplasia was first of a diffuse type, but was later superseded by large so called germinal centers the latter lagging somewhat behind the rise in antibody titer.

The fact that the tissue response accompanying the formation of antibodies was chiefly a lymphocytic one points to the lymphocyte as a factor in the formation of antibodies.

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EXPLANATION OF PLATES

PLATE 26

FIG 1 Rabbit 14 Popliteal lymph node of a normal rabbit showing (a) distinct secondary and (b) pseudosecondary nodules Azur II-eosin $\times 4$

FIG 2 The same, $\times 35$ (a) pseudosecondary nodule, (b) solid secondary nodules, and (c) Flemming's secondary nodule (so called germinal center)

FIG 3 Rabbit 20 Popliteal lymph node 2 days after injection of typhoid vaccine showing diffuse lymphoid hyperplasia with disappearance of secondary nodules Azur II-eosin $\times 4$

FIG 4 The same, $\times 35$

FIG 5 Rabbit 29 Popliteal lymph node 14 days after injection of typhoid vaccine showing marked lymphatic hyperplasia with large confluent so called germinal centers Azur II-eosin $\times 4$

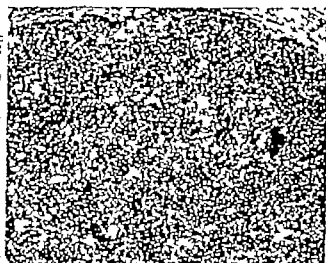
FIG 6 The same, $\times 35$ Compare the size of the secondary nodules in Figs 5 and 6 with those in Figs 1 and 2



1



3



5

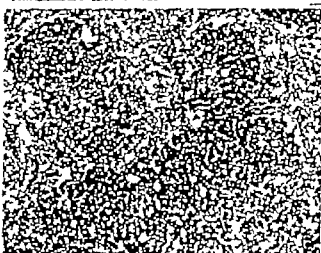


PLATE 27

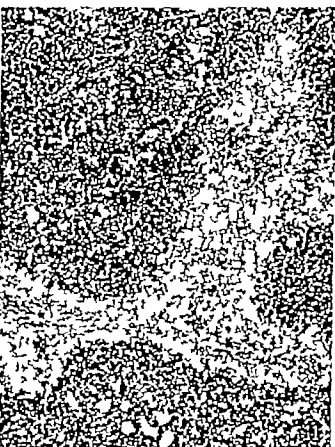
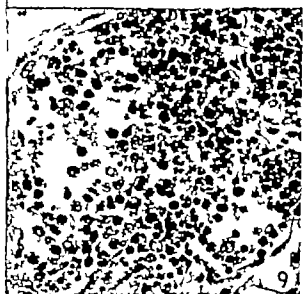
FIG 7 Rabbit 69 Popliteal lymph node 16 hours after injection of typhoid vaccine showing many monocytes in the marginal sinus Azur II-eosin. $\times 270$

FIG 8 Rabbit 52 Popliteal lymph node 6 hours after injection of typhoid vaccine showing (a) outer zone of large lymphocytes around (b) marginal zone of small lymphocytes of Flemming's secondary nodule Azur II-eosin $\times 135$

FIG 9 Rabbit 24 Afferent lymph vessel of popliteal lymph node 24 hours after injection of sheep erythrocytes filled with polymorphonuclear leucocytes, monocytes, and erythrocytes Azur II-eosin $\times 270$

FIG 10 Rabbit 29 Peripheral sinus of popliteal lymph node 14 days after injection with typhoid vaccine filled with large epithelioid cells (reticuloendothelial cells) Azur II-eosin $\times 50$

FIG 11 The same, showing numerous epithelioid cells in a central sinus $\times 50$



SIMULTANEOUS RENAL AND HEPATIC EXCRETION OF WATER, CYANOL, AND AZOFUCHSIN I IN RABBITS*

By WILLIAM E. EHRICH, M.D.

(From the Department of Pathology, The School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication June 11, 1942)

In a previous study of the renal excretion of water, cyanol, and azofuchsin I in rabbits (1), about 90 per cent of the injected amount of azofuchsin I appeared in the urine within 2 hours after injection, while only about 30 per cent of the cyanol was recovered during this time. On the other hand, considerable quantities of cyanol were found in the contents of the intestine, while azofuchsin I was not observed in this location.

Since little is known about the relationship between renal and hepatic excretion of dyes and other substances, and this relationship appears to be important to the student of both renal and hepatic excretion, a study was undertaken of the behavior of the two types of dyes when given simultaneously, and cyanol and azofuchsin I were selected to be studied first because their renal excretion had been investigated previously (1).

Methods

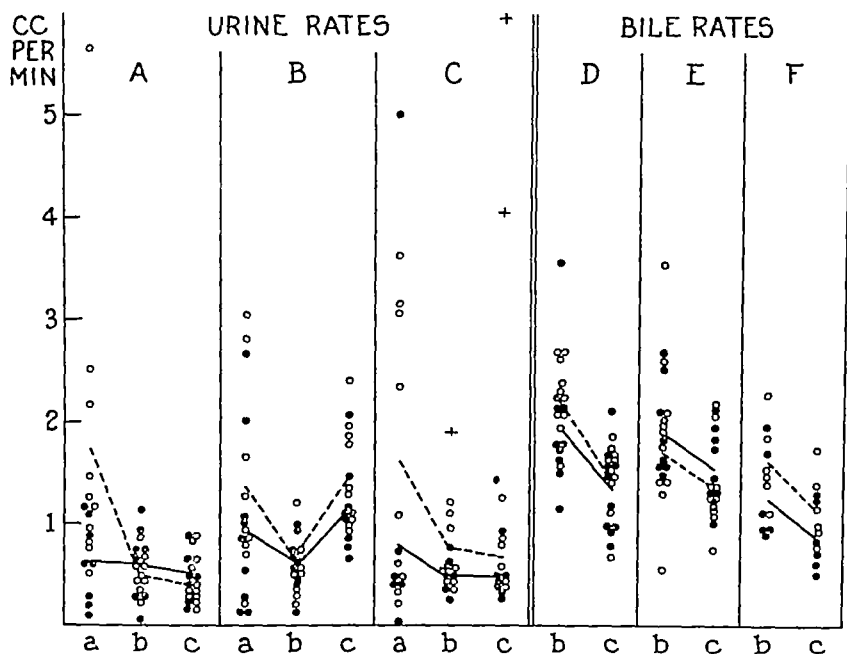
All experiments were performed in male Chinchilla rabbits weighing about 2000 gm. In a first series of experiments the animals received 100 cc. of tap water by stomach tube, and a catheter was placed in the urinary bladder. About 1 hour later when diuresis was in progress the rabbits were anesthetized with ether, and a cannula inserted into the common bile duct. Again, about 1 hour later when the rabbits had recovered somewhat from the anesthesia, 2 cc. of a 0.1 per cent solution of either cyanol or azofuchsin I was injected intravenously, and urine and bile were collected 30, 60, and 90 minutes after the injection of the dye. The concentration of cyanol and azofuchsin I in urine and bile was tested as in our previous experiments by using a comparator (1).

In addition to these simple bile drainage experiments, others were performed in which either the renal or the hepatic excretion was changed by various experimental procedures. Thus in a second series of experiments in order to stimulate diuresis 2 gm. of urea were given intravenously shortly after the injection of the dye. In a third series of experiments the renal arteries and veins were ligated so that renal excretion was stopped completely while in a fourth series the bile was not drained but the common bile duct ligated. In a last small group of animals the common bile duct was ligated and portions of the liver excluded from the circulation by throwing a ligature around the base of one or several lobes of this organ.

* Aided by a grant from the Pennsylvania Chapter of the Society of Sigma Xi.

RESULTS

Text-fig 1 presents the behavior of the rates of the urine and bile flow during the three periods of our experiments, namely (a) before operation, (b) between operation and dye injection, and (c) after injection. The white circles refer to animals which received 100 cc. of tap water by stomach tube the day before the experiment as well as 1 hour before the operation ("wet" animals), while the black circles repre-



TEXT-FIG 1 Urine and bile rates (a) before operation, (b) after operation, but before dye injection, and (c) after dye injection. A and D, simple bile drainage experiments, B and E, urea experiments, C, ligation of common bile duct, and F, ligation of renal arteries and veins. The black circles and solid lines refer to "dry" animals, the white circles and broken lines to "wet" animals.

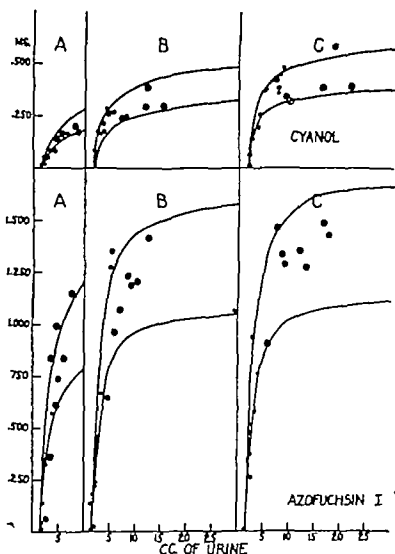
sent results obtained in animals which had water only before the operation ("dry" animals).¹ Both groups of animals had access to drinking water throughout the preoperative period.

Urine Rates—In simple bile drainage experiments (Text-fig 1, A), the average urine rates, instead of rising as in untreated animals, remained low or dropped slightly during the 2nd hour after the introduction of water (between operation and dye injection), to drop even further during the 3rd hour (after dye injection). In the group which received urea after injection of the dye

¹ Both "wet" and "dry" animals were used because in previous experiments wet animals were found to develop a much better water diuresis than dry animals (1).

(B, c) the urine rates rose to more than twice the amount observed in simple bile drainage experiments. If, finally, the common bile duct was ligated (C), the rates closely resembled those in simple bile drainage experiments except in two animals which reacted with a marked increase in urine production (+).

Bile Rates—In simple bile drainage experiments (Text fig 1, D) and in urea experiments (E) the average bile rates dropped from about 0.2 cc. per minute



TEXT FIG. 2. Renal excretion of cyanol and azofuchsin I within (A) 30 (B) 60 and (C) 90 minutes after their injection. The curves indicate ± 20 per cent of the normal average. \circ , simple bile drainage experiments \circ , urea experiments \bullet , ligation of common bile duct.

before dye injection to 0.14 cc per minute after dye injection. In animals in which the renal arteries and veins were ligated (F) the bile rates were markedly depressed. It is noteworthy that the bile rates in our simple bile drainage experiments (D) were about 3 to 4 times the urine rates (A), while after introduction of urea the two were about the same (E, c and B, c).

Excretion of Cyanol and Azofuchsin I—If we turn now to the excretion of the dyes and first consider their renal excretion (Text fig 2), we find that practically all our results are well within ± 20 per cent of the normal excretion

curves² This is true for both the dyes, whether in simple bile drainage experiments (○) urea experiments (○), or those in which the common bile duct was ligated (●) However, if in addition to the ligation of the common bile duct, parts of the liver were excluded from the circulation, the renal excretion of cyanol (the only dye tested in such experiments) was definitely increased (Table I)

As to the hepatic excretion of the two dyes (Text-fig 3), it appears that the results are not numerous enough to permit a conclusion as to whether they conform to definite curves However, it can be seen that in a large majority of experiments the results in the urea experiments (○) tend to be lower than those of the simple bile drainage experiments at comparable bile rates (○), and especially those in which the renal arteries and veins were ligated (●) The greatest amount of cyanol excreted in the bile within 90 minutes after injection

TABLE I

Urine Rates and Cyanol Excretion after Ligation of the Common Bile Duct and Various Portions of the Liver

Rabbit No	Weight	Urne rate			Cyanol excretion (per cent of normal)			Portion of liver ligated
		Before operation	After operation		30 min 60 min 90 min after injection of dye	60 min after injection of dye	90 min after injection of dye	
			Before dye injection	After dye injection				
	gm	cc /min	cc /min	cc /min				per cent
B 12	1800	0 09	0 03	0 08	—	+22	±0	43
B 13	1980	0 02	0 01	0 10	+34	+38	+28	50
B 11	2040	0 17	0 01	0 05	—	+50	+38	67

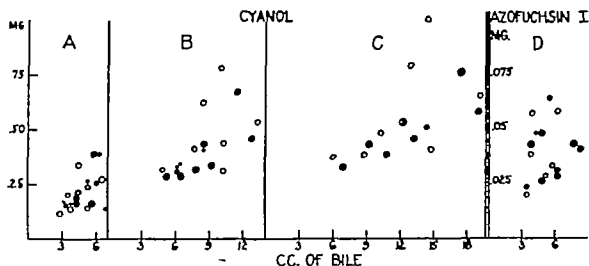
of the dye (C) was about 1 mg, approximately twice the amount observed in the urine during this period, while the greatest amount of azofuchsin I excreted in the bile within 30 minutes³ (D) did not exceed 0 08 mg, or about 1/15th of the largest amount which, during this period, was observed in the urine

It can then be said that after ether anesthesia and drainage of the common bile duct a water diuresis failed to develop, and that ether anesthesia and ligation of the common bile duct in most cases had a similar effect, while 2 gm of urea intravenously definitely stimulated diuresis The excretion of bile, on the other hand, was the same in both simple bile drainage experiments and in those in which urea was given, while in animals in which the renal arteries and veins were ligated the bile flow appeared to be depressed

² The normal excretion curves have been drawn from a large number of data obtained in normal Chinchilla rabbits weighing about 2000 gm About half of these data have been presented in a previous publication (1)

³ After the first 30 minute period the concentration of azofuchsin I in the bile became so low that in many instances it could no longer be determined It is for this reason that the data of the first 30 minutes only are given

It can be stated further that in simple bile drainage experiments,*urea experiments, and those in which the common bile duct was ligated, the usual amounts of cyanol and azofuchsin I were excreted through the kidneys. It is true that the absolute quantities of dye that were recovered from the urine within 30, 60, and 90 minutes after their injection were smaller than those which were found in similar experiments in intact animals (1), however, all the results were well within ± 20 per cent of the normal excretion curves. In other words, there was no true reduction in dye excretion, only the diuresis was depressed. However, if parts of the liver were excluded from the circulation, the renal excretion of cyanol was augmented.



TEXT FIG. 3. Hepatic excretion of cyanol within (A) 30, (B) 60, and (C) 90 minutes, and (D) that of azofuchsin I within 30 minutes after their injection. O, simple bile drainage experiments, O, urea experiments ●, ligation of renal arteries and veins.

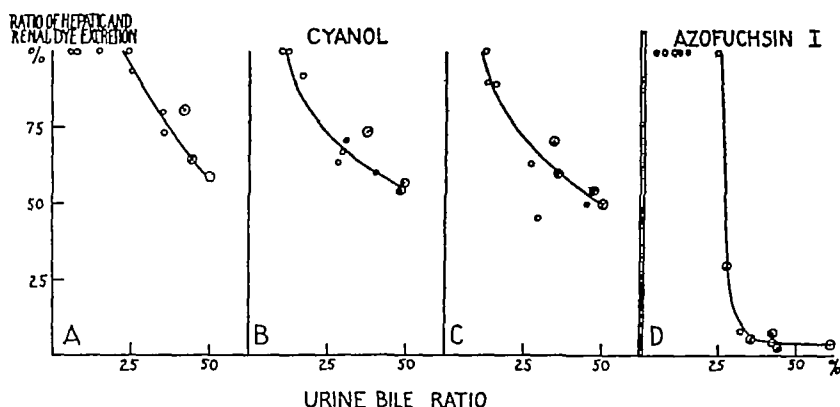
On the other hand, the hepatic excretion of the dyes appeared to be not only a function of the bile volume, but also of their renal excretion, because with the same bile volume the hepatic excretion tended to be slightly greater in animals with a low diuresis than in animals with a better diuresis and it was greatest in experiments in which the renal arteries and veins were ligated.

It thus appears that under the conditions of our experiments the renal excretion of water was independent of its hepatic excretion, and *vice versa* while the renal and hepatic excretion of our dyes were interrelated.

DISCUSSION

Though comparable observations are scanty, the few data which have been published are in good accord with our results. For instance, in rabbits weighing 2000 to 3000 gm Halpert (2) observed an average bile rate of 0.15 to 0.19 cc. per minute during the 1st hour after insertion of a cannula into the common bile

duct, and a rate of 0.12 to 0.17 cc per minute during the 2nd hour, while in our simple bile drainage experiments and those in which urea was given the average bile rate amounted to 0.19 to 0.23 cc per minute during the 1st hour, and 0.13 to 0.155 cc during the following $1\frac{1}{2}$ hours. In obstructive jaundice in man no change was observed in the renal excretion of phenolsulfonephthalein (3, 4). After ligation of the common bile duct in dogs, a slightly elevated output was found by some (5) workers, but a normal or depressed output by others (4), while in our rabbits after ligation of the common bile duct no change in dye excretion was observed. The latter observation may be explained by the assumption that in these cases the liver continued to function, that is to say, it



TEXT-FIG 4 Relationship between urine-bile ratio and that of hepatic and renal excretion of cyanol within (A) 30, (B) 60, and (C) 90 minutes, and (D) that of azofuchsin I within 30 minutes after their injection. The ratios have been calculated as per cent of the total amount excreted. O, simple bile drainage experiments, O, urea experiments.

continued to take up dye so that the amount of dye offered to the kidneys was not materially altered. This is in accord with the fact that after destruction of parts of the liver by chloroform an increase in the renal output of phenolsulfonephthalein was observed (Hanner and Whipple (5)), and a similar increase in the renal excretion of cyanol was noted in our experiments in which, in addition to ligation of the common bile duct, parts of the liver were excluded from the circulation by throwing a ligature around the base of one or several lobes of this organ.

It can be said then that in normal kidneys the renal excretion of a given dye is chiefly a function of the urine volume except in cases of severe destruction of the liver, for then the renal excretion may be augmented. The hepatic excretion of our dyes was found to depend on the urine volume, *i.e.*, the renal excretion of the dyes, as well as on the bile volume, and this was observed even if the

kidneys were normal. This difference can be explained by the fact that diuresis fluctuates a great deal, while the bile rate tends to be more steady. It is obvious that a greater fluctuation in diuresis, and consequently in renal dye excretion, should result in a greater variation in the amount of dye still in circulation, which in turn should bear on the hepatic excretion of the dye. In the kidney, on the other hand, on account of the greater steadiness of the bile rate, this effect would not manifest itself.

In view of these observations it seemed clear that a mathematical relationship between the renal and hepatic excretion of the two dyes could not be found by simply comparing the quantities of dye that were excreted through kidneys and liver. However, when the ratio between the amount of dye excreted through the liver and that excreted through the kidneys was plotted against the ratio between urine rate and bile rate (Text-fig. 4), hyperbolas resulted showing that a logarithmic relationship exists between the renal and hepatic excretion of cyanol and azofuchsin I.

Considering finally the practical question whether or not the hepatic excretion of dyes introduces a noteworthy error into their use as a measure of renal function, we can safely conclude from our experiments that this is not the case except in those instances in which the liver is severely damaged. This conclusion is in accord with the experience of those who use phenolsulfonephthalein for testing the kidney function.

SUMMARY

1 The renal and hepatic excretion of water, cyanol, and azofuchsin I was studied in rabbits under various conditions. It was observed that under the conditions of our experiments the urine rate was independent of the bile rate, and *vice versa*. The renal excretion of these dyes also was independent of their hepatic excretion except in cases in which the liver was severely damaged. On the other hand, the hepatic excretion of these dyes depended also on their renal elimination.

2 The relationship between the renal and hepatic excretion of cyanol and azofuchsin I could be expressed in the form of hyperbolas.

3 In the use of dyes as a measure of renal function, no noteworthy error is introduced by their hepatic excretion except in cases in which the liver is severely damaged.

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INDUCED RESISTANCE OF THE CENTRAL NERVOUS SYSTEM TO EXPERIMENTAL INFECTION WITH EQUINE ENCEPHALOMYELITIS VIRUS

I NEUTRALIZING ANTIBODY IN THE CENTRAL NERVOUS SYSTEM IN RELATION TO CEREBRAL RESISTANCE

By ISABEL M. MORGAN Ph.D. R. WALTER SCHLESINGER M.D., AND
PETER K. OLITSKY M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Neutralizing antibody in cerebrospinal fluid and brain extract of animals vaccinated with the virus of equine encephalomyelitis was studied in an attempt to clarify the apparent lack of correlation of antibody with resistance to intracerebral injection of active virus. Hurst (1) has reported that monkeys, after peripheral injection of active virus of Eastern equine encephalomyelitis (E.E.E.), developed neutralizing antibody. Certain of these animals succumbed, nevertheless, to subsequent intracerebral injection of active virus. The neutralization tests were carried out with undiluted serum. Such tests with undiluted serum have been found (2), however, incapable of differentiating, above a certain level, between the neutralizing capacity of different sera. When serum was titrated for antibody, using dilutions of serum and a constant amount of virus, it was found that resistance of vaccinated mice to intracerebral injection of active E.E.E. virus was associated only with a high level of antibody in the serum. The degree of resistance was correlated with antibody titer. Since the plasma does not have direct access to cells of the central nervous system, the high titer of antibody in the plasma suggested that it might be an indicator of antibody in the fluids of the central nervous system which would be more readily available to susceptible cells.

Antibody has been found by Freund (3) in the brain, spinal cord, and cerebrospinal fluid of rabbits vaccinated with typhoid vaccine. He has demonstrated, moreover, a constant relationship between the concentration of antibody in the central nervous system and that in the serum. Ramon and Descombey (4) have described a similar relationship of antitoxin in spinal fluid and serum. Mollaret and Stefanopoulo (5) have reported that monkeys surviving a subcutaneous injection of active yellow fever virus showed neutralizing antibody in their spinal fluid and resisted an intracerebral injection of active virus.

In rabbits vaccinated with equine encephalomyelitis virus we studied first, the relationship of antibody in spinal fluid to that in serum and second, its bearing on cerebral resistance to active virus.

Methods and Materials

Rabbits—Rabbits of various breeds weighing from 2500 to 3500 gm were obtained from dealers in the vicinity. They were usually kept in individual cages in the animal house for a week before use. For each group of test animals, controls were saved from the same lot. There were many animals used in addition to those which appear in the tables on which the record was not complete, but which, as far as tested, were consistent in their reaction with the others. One lot of animals had been bred and raised in our animal house, they will be mentioned again because of their unusual reaction.

Rabbits were vaccinated, usually subcutaneously, with active or formalin-inactivated Eastern (E E E) or Western (W E E) equine encephalomyelitis virus. The source of active virus (A V) was usually a saline suspension of infected 9-day-old chick embryo (sometimes infected mouse brain). Formalin-inactivated virus (F V) consisted of a 10 per cent suspension of infected chick embryo in 0.5 per cent formalin, which was non-infective by all tests available (6).

Cisternal Puncture—Cerebrospinal fluid was obtained from the cisterna magna of rabbits anesthetized by intravenous injection of nembutal. An assistant firmly grasped the head of an anesthetized rabbit over the edge of a table with the top of the head vertical, the neck thus flexed at a right angle. A 22-gauge, 1½-inch needle was then inserted in the midline, just caudal to the occipital protuberance. When in the proper position, with the needle at a depth of about ½ inch, the piercing of the dura could be easily felt and, when spinal fluid welled up into the hilt of the needle, it was drawn off with a capillary pipette. About 0.5 cc. of spinal fluid could be taken without risk. Only crystal clear spinal fluid was acceptable, in which no red blood cells were detectable after standing overnight in the refrigerator. Contamination with blood was more easily detected in this way than by cell count, for cells were visible in a slightly contaminated sample which showed less than one cell per microscopic field. With practice we were able to obtain clear spinal fluid from more than half the number of rabbits. Control rabbits were tapped at the same time as the test animals.

Tests for Resistance—Tests for cerebral resistance of vaccinated rabbits were carried out by intracerebral or intracisternal injection of saline or broth dilutions of a suspension of mouse brain infected with W E E or E E E virus. Non-vaccinated rabbits from each lot served as controls of the infectivity of the virus.

Intracerebral Route—A small hole was drilled with a trephine caudally to the frontoparietal suture to one side of the midline. 0.2 cc. of viral suspension was injected through a 27-gauge needle, ¾ inch long, inserted vertically to the hilt. In this way, most of the virus injected went into the lateral ventricle, as confirmed by examination of brains immediately after injection of a dye (mixture of iron ammonium citrate and potassium ferrocyanide) with formalin.

Intracisternal Route—A syringe containing suspension of virus was attached to the needle through which a sample of spinal fluid had been removed, thus injecting the virus directly into the cisterna magna whence it had free access to the entire sub-arachnoid space. In this way, when a clear sample of spinal fluid had been obtained, the virus did not come into contact with blood or with damaged cerebral tissue.

EXPERIMENTAL

Neutralizing Capacity of the Cerebrospinal Fluid

The spinal fluid of rabbits vaccinated with active or with formalin inactivated virus was tested for neutralizing property

Samples of blood and spinal fluid were taken from rabbits after subcutaneous vaccination with one or more doses of active or formalized W.E.E. or E.E.E. virus. Samples were taken, as a rule 2 weeks after the last dose of vaccine. Neutralization tests were carried out by the peritoneal test in young mice (7), using infected mouse brain. When testing for minimal amounts of antibody the peritoneal test proved to be more sensitive than the cerebral neutralization test. Tests on spinal fluid and dilutions of serum of a given animal were made simultaneously and repeated whenever possible.

In the spinal fluid of rabbits sufficiently vaccinated, the capacity to neutralize the homologous virus could be readily demonstrated. Furthermore, when the neutralizing capacity of threefold dilutions from 1/10 to 1/1000 of serum of a given animal was compared with that of its spinal fluid, a dilution of serum of the order of 1/300 was found equivalent to spinal fluid. Table I shows the neutralizing capacity (as measured by difference in titer of virus in the presence of normal spinal fluid and that in test fluid) of spinal fluid and 1/300 dilution of serum of vaccinated rabbits.

In all cases but one, shown in Table I, when 1/300 serum dilution neutralized virus, so also did the spinal fluid when one failed to neutralize so did the other. There was not more than a tenfold difference in neutralizing capacity when end points were reached.

In the single exception, rabbit 35 the spinal fluid failed in a single test to neutralize virus, whereas the 1/300 serum dilution neutralized at least ten units of active virus on repeated test. This animal was from the group of cage-bred rabbits.

Just as has been reported for the ratio of antibody in spinal fluid to that in serum in response to a bacterial antigen (3) and to toxin (4), so also a ratio of neutralizing capacity of spinal fluid to serum of the order of 1/300 has been demonstrated in animals vaccinated with a viral antigen.

Neutralizing Capacity of Brain Tissue

The neutralizing capacity of a suspension of perfused brain of a rabbit vaccinated with formalin inactivated W.E.E. mouse brain was compared with that of its spinal fluid and 1/300 serum dilution.

Rabbit 2-32 was vaccinated by means of thirteen subcutaneous injections of 2 cc. or more of 20 per cent mouse brain, infected with W.E.E. virus in 0.5 per cent formalin over a period of 3 months. 5 days after the last dose, clear spinal fluid was obtained.

75 cc of blood were drawn from the heart. The brain was perfused by injection of sterile saline through the carotid arteries until the fluid returning from the cut veins appeared blood-free. 500 cc of saline were injected on one side, followed by 100 cc. on the other. A piece of brain was sectioned for histological study which revealed

TABLE I
Neutralizing Capacity of Spinal Fluid Compared with 1/300 Serum Dilution of Rabbits Vaccinated with E E Virus

Rabbit No	Vaccination	Neutralizing capacity	
		1/300 serum	Spinal fluid
22 91	36 × A V -W E E *	1,000	1,000
10-06	5 × A V -E.E.E	100-1,000	>100†
10-02	5 × A V -E E E	1,000	>100
10-39	3 × A V -W E E §	1,000	1,000
10-42	3 × A V -W.E.E	1,000	1,000-10,000
10-03	3 × A V -E E E	>10	10
10 14	3 × A V -E E E	10	>10
2-32	13 × F V -W E E	100	10-100
1-56	3 × F V -W E E	10-100	>10
1-57	3 × F V -W E E	10	>10
79 A	1 × A V 10 ⁻² -W E E	>100	>100
33	1 × A V 10 ⁻⁵ -W.E.E	10-100	10-100
35	1 × A V 10 ⁻⁵ -W E E	>10	0
83 A	1 × A V 10 ⁻⁵ -W E E	0	0
1-30	3 × F V -W E E	0	0
2-00	3 × F V -W E E	0	0
2-03	3 × F V -W.E.E	0	0
78 B	1 × F V 10 ⁻¹ -W.E.E	0	0
87 B	1 × F V 10 ⁻¹ -W E E	0	0
80 B	1 × F V 10 ⁻² -W E E	0	0
81 B	1 × F V 10 ⁻² -W E E	0	0

* 36 × A V -W E E = 36 injections by intravenous or subcutaneous route of active W E E virus, 10 to 20 per cent. The next two animals, 5 injections similarly of E E E virus

† >100 = difference in titer between control and test was at least 100-fold

§ 3 × A V -W.E.E = 3 subcutaneous injections, all other animals injected by same route, F V = formalized virus

|| Vaccinated with mouse-brain virus, all others with chick-embryo virus

few red blood cells in the vessels. The remainder of the brain was ground in a mortar and made to a $\frac{1}{8}$ suspension in saline. On centrifugation of this cream-colored suspension, no layer of red blood cells was visible. The supernate was drawn off and compared with spinal fluid and 1/300 serum dilution by repeated peritoneal neutralization tests (?) with mouse-brain W E E virus.

From a vaccinated rabbit 1/300 serum dilution, 1/3 suspension of perfused brain, and undiluted spinal fluid each neutralized about ten units of W E E

virus. This is similar to the finding of Freund that the ratio of titer of serum is to that of brain and to that of spinal fluid as 300:2.5:1.

Specificity of the Neutralizing Substance

Spinal fluid of an animal vaccinated with Western virus which could neutralize Western virus was not capable of neutralizing Eastern virus and *vice versa*. Since the neutralizing property of spinal fluid developed in response to vaccination parallels the serum antibody in the ratio described, and since the neutralizing substance reacted specifically with the homologous and not with the heterologous virus, it was considered antibody (6, 8, 9).

Correlation of Spinal Fluid Antibody with Resistance to Infection by the Cerebral Route

(a) *In Response to Active Virus*—The spinal fluid (or 1/300 serum dilution) of thirty rabbits vaccinated subcutaneously with various doses of active chick embryo virus was tested for neutralizing antibody to the homologous virus by the peritoneal neutralization test in young mice (7). The vaccinated rabbits, as well as normal controls, were injected intracerebrally with active, homologous, mouse-brain virus. The results are summarized in Table II.

Twenty-two rabbits showed neutralizing antibody in the spinal fluid (or 1/300 serum dilution), (Table II). Twenty-one of these survived an intracerebral injection of active virus in a dose 10 to 100 times the highest dilution lethal to normal controls. All eight rabbits having no demonstrable spinal fluid antibody succumbed after a typical course following intracerebral injection of active virus.

The single exception—an animal which died in spite of the presence of spinal fluid antibody, occurred in the cage-bred group. It may be recalled that it was in this group also that the single exception to the equivalence of spinal fluid antibody to 1/300 serum antibody was found.

It may be seen from Table II that rabbits reacted to subcutaneous injection of active virus with either a high degree of immune response, with antibody in the spinal fluid and with resistance to an intracerebral injection of active virus, or else there was no antibody demonstrable, even in undiluted serum, and no cerebral resistance. However, even the immune animals were not entirely refractory; they reacted to intracerebral injection of active virus with febrile response lasting a few days. In general, animals with higher titer of spinal fluid antibody showed fever of shorter duration. This febrile response was interpreted as due to viral activity because the greater the test dose of virus, the greater the febrile spike on the first day following test injection, and because intracerebral injection of formalin inactivated virus (at high concentration) induced no such reaction, even in normal animals. As might be ex-

pected, the full immune response occurred more frequently following multiple doses of virus or a large single dose. Thus it has been shown that cerebral resistance induced as a result of vaccination with active virus is associated with spinal fluid antibody.

The significance of this antibody which develops after vaccination with active virus is, however, not entirely conclusive, for (a) After peripheral inoculation of active virus, the systemic phase of infection, accompanied by circulating virus, may be followed by a transitory invasion of the central nervous

TABLE II

Correlation of Spinal Fluid Antibody with Resistance to Intracerebral Injection of Active Virus in Rabbits Vaccinated Subcutaneously with Active Virus

Total No of rabbits vaccinated	Vaccination subcutaneously	Antibody			Reaction to intracerebral injection	
		Serum		Spinal fluid		
		Undiluted	1/300		Fever	Outcome
2	36 X W.E.E		+	+	1-2 d	Recovery*
2	5 X E.E.E	+	+	+	1-5 d	"
3	3 X E.E		+	+	1-2 d	"
2	1 X 10 ⁻¹ W.E.E		+	+	0-1 d	"
2	1 X 10 ⁻² W.E.E	+	+	+	1-3 d	"
4	1 X 10 ⁻³ W.E.E		+	+	0-1 d	"
3	1 X 10 ⁻⁴ W.E.E		+	+	0-1 d	"
3	1 X 10 ⁻⁵ W.E.E		+	+	0-2 d	"
1	1 X 10 ⁻⁵ W.E.E	+	+	+	4 d	Death
1	1 X 10 ⁻⁵ W.E.E	+	+	0	4 d	"
2	1 X 10 ⁻² W.E.E	0	0	0	2 d	"
1	1 X 10 ⁻⁵ W.E.E	0	0	0	4 d	"
4	1 X 10 ⁻⁷ W.E.E	0		0	2-3 d	"

* 1-2 d Recovery = 1 to 2 days of fever, followed by recovery, other symbols same as in Table I

system, reflected by a marked rise in temperature, although the animal may show no clinical signs of involvement of the central nervous system and recover with full antibody response (10). (b) Following intracerebral injection of small doses of active virus, rabbits may also show only a transitory febrile reaction and may not develop demonstrable antibody. Such individuals subsequently resist intracerebral inoculation of the same virus, or even of heterologous virus. This type of non-specific resistance, independent of antibody, will be discussed in the next paper of this series (11). The possibility cannot be ruled out that a non-specific effect may play a part in the cerebral resistance after vaccination with active virus. Therefore we undertook vaccination with formalin-inactivated virus where by all tests available the possibility of the presence of active virus has been excluded.

(b) *In Response to Formalin Inactivated Virus*—In contrast to the "all or none" immune response induced by active virus, it was possible to obtain intermediate degrees of immune response only when we resorted to vaccination with formalin inactivated virus

TABLE III

Antibody Response and Resistance Induced by Formalin-Inactivated W.E.E. Virus

Rabbit No.	Vaccination subcutaneously	Antibody				Test for resistance		
		Serum			Spinal fluid	Route	Fever	Outcome
		Undiluted	1/200	1/300				
2-89	7 × 20 per cent*			+	+	Intracerebral	3 d.	Recovery
1 56	3 × 10 "			+	+	Intracisternal	0	"
1 57	3 × 10 " "			+	+		2 d.	
1 53	3 × 10 "			+			3 d.	
1-30	3 × 10 "		+	0			2 d.	
1 24	1 × 10	+	0	0			4 d.P.P.†	
1 27	1 × 10	+	0				5 d.	
94	1 × 10	+	+	0		Intracerebral	3 d.	Death
97	1 × 1	+	0	0			3 d.	
87 B	1 × 10	+	0	0	0		3 d.	
2-00	3 × 10	+	0	0	0		3 d.	
2-03	3 × 10	+	0	0			3 d.	
1 54	3 × 10 "	+	0	0		Intracisternal	6 d.	
1 55	3 × 10	+	0	0			2 d.	
1 18	1 × 1 "	+	0		0		3 d.	
1 19	1 × 1	+	0		0		3 d.	
1 25	1 × 1	+	0		0		2 d.	
1 28	1 × 1	+	0				5 d.	
1 29	1 × 1	+	0		0		5 d.	

* 7 × 20 per cent = vaccinated by 7 subcutaneous injections of formalized, 20 per cent chick-embryo vaccine of W.E.E. virus

† 4 d.P.P. = 4 days of fever with posterior limb paralysis.

Rabbits were vaccinated by one or more subcutaneous injections of chick-embryo W.E.E. virus inactivated by formalin. Samples of spinal fluid and blood were taken usually 2 weeks after the first dose. Cerebral resistance was tested either by injection of active mouse brain virus into the cisterna magna through the needle used for withdrawal of fluid or by intracerebral injection on the following day. Antibody in the spinal fluid and in serum dilutions was measured by the peritoneal neutralization test (7). Fluids in a given series were tested simultaneously. The results are shown in Table III.

It may be noted from Table III that it is possible to induce resistance to intracerebral injection of active virus in rabbits by vaccination with formalin-inactivated, chick-embryo WEE virus. Similar results were obtained using FV-mouse-brain vaccine. Individuals which had developed antibody demonstrable in spinal fluid and in 1/300 serum dilution survived an intracerebral or an intracisternal injection of active virus. Temperatures read daily following test injection were normal or indicated fever lasting up to 3 days. At the other extreme, when antibody could not be demonstrated in the spinal fluid, such injection of active virus led to death from typical encephalitis, in spite of demonstrable antibody in the undiluted serum.

Intermediate between these two groups were individuals, like rabbit 1-30, which showed antibody at 1/200 but not at 1/300 dilution of serum and recovered after 2 days of fever. In this case, no sample of spinal fluid was available, but since 1/300 serum dilution did not reveal antibody, our experience indicates that an equal volume of spinal fluid would also be negative, however, since the serum at only a slightly higher concentration did show antibody, so also a larger sample of spinal fluid might be expected to be positive. The demonstration of antibody at 1/200 serum dilution in this individual may be contrasted to its absence at the same dilution of serum of rabbits 1-24 and 1-27 of the same series, which, although they survived, showed a prolonged course of fever, and even posterior limb paralysis in one. Also in the same series were rabbits 1-18 through 1-29, with no antibody at 1/200 serum dilution and no cerebral resistance. Two additional rabbits of interest are 94 and 97, vaccinated simultaneously with different doses, the former showed antibody at 1/200 serum dilution and was resistant, the latter did not, and succumbed.

The immune response and result of subsequent intracisternal injection of active virus in the series of rabbits 1-53-1-57 are shown in Fig. 1.

The three rabbits which showed antibody in the 1/300 serum dilution, and in spinal fluid when tested, resisted the test dose, whereas the two in which no antibody was demonstrable in the 1/300 serum dilution succumbed to it.

In summary, rabbits which had antibody demonstrable in the spinal fluid were found to resist active virus introduced into the central nervous system, lack of spinal fluid antibody was associated with lack of such resistance.

Specificity of Immunity Induced by Vaccination with Formalin-Inactivated Virus

Rabbits vaccinated with formalin-inactivated Western EE or Eastern EE virus were tested for cerebral resistance to Western EE virus.

Three rabbits were vaccinated by seven subcutaneous injections of 2 to 5 cc. of 20 per cent suspension of chick-embryo EEE virus inactivated by formalin. Three rabbits were vaccinated with formalized WEE virus. Six doses were given on alternate days and after a rest period of 4 weeks, a final dose was given 5 days later, samples of blood and spinal fluid were taken and on the following day, the animals were injected intracerebrally with dilutions of mouse-brain WEE virus. From six normal control rabbits, spinal fluid was obtained at the same time and they were injected with amounts of virus indicated in Fig. 2.

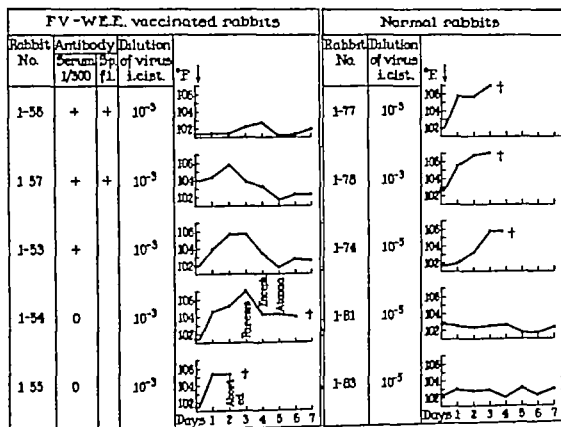


FIG 1 Correlation of antibody in spinal fluid (or in 1/300 serum dilution) of FV vaccinated rabbits with resistance to intracerebral injection of active virus

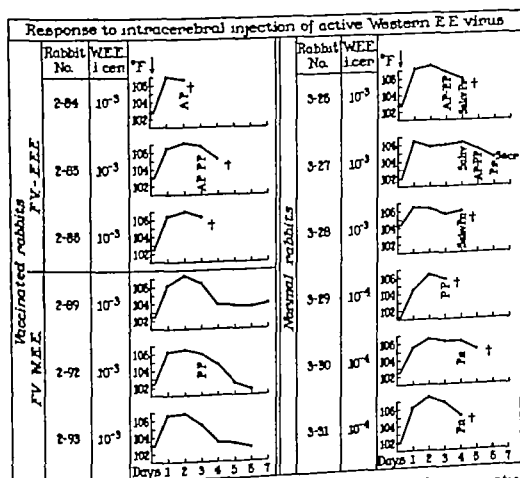


FIG 2 Specificity of cerebral resistance induced in rabbits by vaccination with formalized Eastern or Western E.E. virus. AP = anterior limb paralysis PP = posterior limb paralysis, Pr = prostrate.

All normal rabbits succumbed to intracerebral injection of active W E E virus in dilutions indicated in Fig 2, after showing signs of typical encephalitis. Of the vaccinated rabbits, the three vaccinated with Eastern virus succumbed typically, while the three Western virus-vaccinated animals showed a normal temperature after 3 to 4 days of fever, and recovered. In a reciprocal test, rabbits vaccinated with F V - W E E virus succumbed to active Eastern, but resisted Western virus.

Thus cerebral resistance to virus was induced in rabbits by vaccination with formalin-inactivated vaccine of the homologous virus but not by the heterologous virus. This is in agreement with consistent evidence that Eastern and Western E E viruses are immunologically distinct, showing no cross-reactions, either by serum-neutralization test (8, 9) or by resistance tests in immune animals (6, 11).

DISCUSSION

It has been found that a definite ratio exists between the concentration of neutralizing antibody in the serum, brain tissue, and cerebrospinal fluid of rabbits vaccinated with equine encephalomyelitis virus. This confirms the earlier observation of Freund on agglutinin to typhoid bacilli. The results here reported were based chiefly on tests with spinal fluid rather than perfused brain extract, because (1) we could be more certain that there was no contamination with plasma, (2) the animal could be spared for resistance test, and (3) the concentration of antibody in the perfused brain extract was fully as high as that in the spinal fluid.

Whether spinal fluid obtained from the cisterna magna represents a fluid similar to intercellular fluid of the brain is still under discussion. Besides the well known source of spinal fluid in the choroid plexuses of the ventricles (12, 13, cf 14), there is a possible extraventricular source. According to the experiments of Weed (15), there is a direct continuity of the intercellular spaces of the brain cells with pericapillary spaces and these in turn with the Virchow-Robin spaces surrounding the pial vessels and finally the subarachnoid space. Furthermore, he demonstrated a flow in the direction named, *etc.*, from the capillaries to the subarachnoid fluid. Kafka (13) presents diagrams of this circulation. Since in mammals there is no evidence of a lymphatic system within the central nervous system, the circulation described may be considered analogous to that of the interstitial fluid of other tissues. Slight differences in chemical composition between ventricular fluid and subarachnoid fluid lend supporting evidence for an extraventricular formation of spinal fluid and for such a physiological function. Friedemann (19), on the other hand, has reviewed the evidence for his belief that the spinal fluid is not necessarily an intermediary between blood and brain cells.

Regardless of the relationship between the interstitial and the cisternal

fluids, antibody in the spinal fluid serves as an indicator of antibody in the brain tissue, since there is a constant ratio between them

The finding of antibody in the spinal fluid in a constant, but low ratio to that in the serum is in accord with the recent report of Kabat, Landow, and Moore (16), that the proteins of concentrated human spinal fluid show an electrophoretic pattern similar to that of the plasma proteins, and that alterations in the composition of serum proteins produce similar changes in the spinal fluid patterns. The ratio reported of antibody in the spinal fluid to that in the serum is, moreover, similar to the total protein ratio (Flexner (17) gives an average total protein ratio of 1:250, although the normal range in both fluids is wide.) A similar ratio of agglutinin in spinal fluid to that in serum was established in animals which received antiserum intravenously, i.e., by passive immunization (3). For these reasons, we believe that there is no necessity for assuming local production of antibody (18), but that antibody in the spinal fluid merely reflects the antibody in the plasma in the ratio described.

In an animal sufficiently vaccinated, antibody is present in the central nervous system, as determined by tests on spinal fluid. Such an animal survives an intracerebral injection of active virus which is lethal for non vaccinated controls. That some cells may become infected, nevertheless as an immediate consequence of intracerebral injection of active virus despite the presence of antibody in the central nervous system, is indicated by the febrile response following the injection. The mode of action of the antibody in the central nervous system may then be to neutralize virus deriving from infected cells and thus to prevent the spread of infection. Also, a certain amount of virus injected into a ventricle may be neutralized by the antibody present in the spinal fluid. By the experimental procedure of an intracerebral injection, virus is placed in direct contact with susceptible cells, in contrast to inoculation of a vaccinated animal by a peripheral route where antibody may have full access to the virus before it reaches the central nervous system (6).¹

The significance of antibody in the course of infection following peripheral inoculation of virus (probably simulating more closely the natural mode of infection) has been discussed (10). After subcutaneous injection of active W.E.E. virus in adult rabbits, systemic infection, accompanied by virus in the blood stream, may be followed by virus invasion of the central nervous system, characterized by high fever but without apparent signs of involvement of the nervous system. Such infection takes place when antibody has already appeared in the blood stream. Defervescence and recovery

¹ H. B. Shumacker Jr, and associates (*J Immunol*, 1939 87, 425 *Bull Johns Hopkins Hosp*, 1940 67, 92) have reported that in active and passive immunization in different species of animals, a higher level of antitoxin is needed for protection against tetanus toxin introduced into the spinal cord or medulla than when the toxin is given peripherally.

set in at a time when serum antibody titer reaches 1/300 (or, in other words, when antibody is present in the spinal fluid) On the other hand, young rabbits, after a similar injection of virus, die of encephalitis before this antibody level has been reached

In human beings convalescent from infection with W E E virus, Howitt (20) found neutralizing antibody in the spinal fluid of seventeen of twenty cases which showed serum antibody In four monkeys which survived infection induced by intracutaneous injection of W E E virus, antibody appeared in the spinal fluid

It has been said that the central nervous system is impervious to antibody, because a vaccinated animal may show antibody in the serum and yet not resist an intracerebral injection of a neurotropic virus The same apparent lack of correlation has led some to believe that antibody has nothing to do with immunity of the central nervous system Having defined the conditions for availability of antibody, we believe that the rôle of antibody in immunity of the central nervous system is not unique but differs quantitatively from that of other tissues, *i e*, that it is necessary to have a high titer of antibody in the plasma in order to have a minimal amount present in the central nervous system The concept of availability of antibody may be applied to immunity to other infections Francis (21) has reported that serum antibody in influenza patients rose between the acute and convalescent phases, accompanied by a rise, at a considerably lower level, of inactivating capacity of the nasal secretion He suggested the possibility that only those individuals essentially devoid of such inactivating substance in their nasal secretions need be considered susceptible to infection with the virus

This definition of the relation of antibody to resistance allows immunity of the central nervous system to fall in line with the generally accepted principles of immunology

SUMMARY

1 Neutralizing antibody to equine encephalomyelitis virus was found in the spinal fluid of rabbits sufficiently vaccinated with active or formalin-inactivated virus Antibody was specific for the Western or for the Eastern virus

2 Neutralizing capacity of spinal fluid was equivalent to that of a 1/300 dilution of serum of the same animal, and was of the same order of magnitude as that of perfused brain of a vaccinated animal

3 Vaccinated rabbits which showed antibody in the spinal fluid resisted intracerebral or intracisternal injection of active virus This immunity was specific, *i e*, there was no cross-reaction between the Eastern and Western virus after vaccination with formalin-inactivated virus On the other hand, lack of antibody in the spinal fluid, even when antibody was demonstrable in the undiluted serum, was associated with lack of cerebral resistance

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THE CONTROL OF THE RENAL EXCRETION OF WATER*

I THE EFFECT OF VARIATIONS IN THE STATE OF HYDRATION ON WATER EXCRETION IN DOGS WITH DIABETES INSIPIDUS

By JAMES A. SHANNON M D

(From the Departments of Physiology and Medicine, New York University College of Medicine, and the Research Service, Third (New York University) Medical Division Welfare Hospital New York)

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Chronic polyuria following ablation of the neurohypophysis in the mammal is presumably due to a lack of adequate amounts of the posterior pituitary antidiuretic hormone. The polyuria of this condition has itself received considerable attention. What has not been commonly appreciated is the wide fluctuations to which it is subject and which are commonly the result of variables known to influence the water excretion of normal animals. The present investigations examine into the circumstances which surround the effect of variations in bodily hydration on the rate of water excretion in diabetes insipidus. The variations in hydration were produced by the administration and withdrawal of water and by the ingestion of saline solution. The experiments were designed to satisfy two ends. The first of these was a functional description of the potentialities of the nephron to reabsorb water in the absence of the antidiuretic hormone. The second was the clarification of the relationships among the individual processes within the nephron which together determine the renal excretion of water.

EXPERIMENTAL

Material—Four female dogs with permanent diabetes insipidus were used. They were prepared for us by Dr. Allen Keller of the University of Alabama and were maintained in his laboratory for at least one year after the operation. The animals were fed a synthetic diet¹ during their stay in this laboratory permitting easy control of the foodstuff mixture, its caloric value, and the salt content. The caloric intake

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¹ The basic diet consisted of

100 gm. cracker meal	35 gm. meat residue
30 gm. milk powder	30 ml. cod liver oil
10 gm. brewers yeast	3.0 gm. salt mixture

The salt mixture consisted of

4.0 gm. magnesium citrate	10.0 gm. sodium chloride
1.0 gm. ferric citrate	4.0 gm. calcium phosphate

was adjusted so that each animal approximately maintained its weight during the 18 months of the present study. Dog D died suddenly of an acute suppurative hepatitis after some months of apparent good health. Dog C had two attacks of water intoxication. They were promptly relieved by the intravenous administration of hypertonic salt solution and appeared to leave no ill effects. The animals were otherwise in good apparent health.

The operative procedure to produce the diabetes had been somewhat different in the animals. The pituitary stalk was separated from the hypothalamus anteriorly in dogs A and B, and completely in dogs C and D, and a variable amount of contiguous anterior hypothalamic tissue was destroyed. At autopsy, the striking abnormalities were neurological except for a moderate diminution in the volume of the suprarenal cortex and, in dog D, the hepatitis noted above (1). The pars nervosa of the posterior pituitary was completely absent in dogs C and D, while small remnants of the infundibulum, completely separated from the anterior hypothalamus, remained in dogs A and B. It was estimated that there were approximately 1 to 2 per cent of the normal number of cells remaining in the supraoptic nuclei in dogs C and D, 10 per cent in dog A, and slightly more than this in dog B. This evaluation was based upon the examination of every 5th or 10th section from serial sections of the area. Complete cell counts of the supraoptic nuclei did not seem warranted for the purposes of the present study. In all cases the anterior pituitary had an abundance of normal tissue remaining.

A severe functional deficiency was present in each animal (Table I), and it was because of this functional evidence that the animals were selected for study. However, the anatomical findings serve a useful purpose. They lend support to previous work (2) which indicates that, while the supraoptic-hypophyseal system may be necessary for normal water balance, its complete destruction is not essential for the establishment of a permanent polyuria (dogs A and B). If the current view that the posterior pituitary hormone arises exclusively in the cells of the neurohypophysis (3) is correct, they minimize the possibility that any antidiuretic hormone was available in either dog C or dog D.

Methods—The experimental routines are described briefly in the text or by reference. During short experiments the animals were placed upon a comfortable animal board. They were trained so that restraints were unnecessary and they were never supine for any considerable length of time. During longer experiments they were kept in large metabolism cages.

Glomerular filtration rate was measured by the creatinine or inulin clearance. Creatinine was determined by the Jaffé reaction, and inulin by copper reduction subsequent to acid hydrolysis (4). An alkaline copper solution was used which is suited to low concentrations of hydrolyzed inulin and which is adaptable to the Evelyn photoelectric colorimeter. It is necessary for the plasma and urine hydrolysates to be neutral and to have a negligible buffer capacity when this reagent is used (5). Freezing point determinations were made by a method well suited for small volumes of the fluid (6). Total base was determined by electrodialysis (7), sodium by uranyl zinc acetate precipitation following a preliminary dry ashing (8), chloride by silver precipitation (9), total solids of plasma and plasma water by weight, urea by manometric measurement (10). Extracellular fluid volume was approximated

or measured by sulfocyanate or bromide (11) Changes in total solids of plasma were accepted as accurate reflections of changes in plasma volume if the latter were produced acutely (Tables VI and VII) The validity of this relationship in the longer experiments (Tables IV and V) is open to question

RESULTS

Daily Water Balance.—The data of Table I are illustrative of the extent of chronic polyuria in the four animals when they had free access to drinking water The observations were made shortly after the arrival of the animals in this laboratory and at least one year after the onset of permanent polyuria The polyuria remained of the same order of magnitude during the remainder of their stay save when variables were introduced which specifically affected it (cf Table V and footnote 3) The activity of the animals was somewhat

TABLE I
Summary of Water Exchange during First 2 Months of Observation

Dog	Operated upon	Observation	Weight	Surface area	Water intake		Urine output		Glomerular filtration rate	
					Minl. mum	Maxl. mum	Minl. mum	Maxl. mum	Minl. mum	Maxl. mum
			kg	m ²	liters/day	liters/day	liters/day	liters/day	ml./min.	ml./min.
D	Aug., 1938	Nov., 1939	8.80	0.50	3.97	6.07	3.85	5.61	22.4	34.9
A	Mar. 1938	Sept. 1939	11.80	0.55	3.02	5.48	2.67	5.34	36.1	53.8
B	May 1938	Sept. 1939	7.25	0.43	3.10	6.31	2.87	6.01	26.4	41.2
C	July 1938	Nov. 1939	11.30	0.55	3.24	4.96	3.06	4.50	24.0	33.0

restricted during this time since the collection of the data required the use of metabolism cages The experiments which sampled the glomerular filtration rate were so designed that they did not seriously influence the water balance of the animals.

The renal excretion of water was not closely correlated with body weight, surface area, or rate of glomerular filtration, nor with the extent of neurological injury The latter finding is particularly emphasized by the data on dog B This animal was the smallest of the four and the destruction of the supraoptic hypophysial system, as judged by cells remaining in the supraoptic nuclei, was less than in the remaining three and, more particularly, than in dogs C and D However, dog B had a greater polyuria throughout all periods of study than any of the other animals. Such variability is perhaps to be expected in a small group of animals. It would be surprising if the extent of the polyuria in any animal was not conditioned considerably by the sensitivity of the thirst mechanism as well as by the operation of other factors which influence the water balance of normal animals

The detailed data indicate that a relatively constant water turnover on consecutive days does not imply a similarly constant rate of urine formation throughout the day. When the day was divided into two 12 hour periods, the initial one starting at the time the animals were fed, the water intake and the rate of water excretion were considerably higher during the initial than during the subsequent 12 hour period. The urine output during the second 12 hour period was usually well below one-third of the total water excretion for the day. In this respect the animal with diabetes insipidus does not differ greatly from the normal animal (12) and it appears as if a normal mechanism is simply superimposed upon the elevated water exchange. The presence of salt in the diet may be expected to contribute to the effect and to have a simple explanation (see page 378), though multiple factors are undoubtedly involved (13, 14).

TABLE II

Experiments Which Examine into the Relationship between the State of Hydration and the Glomerular Filtration, Urine Flow, and Tonicity of Urine

Dog	Free access to water			After 40 ml per kg water			After 18 hrs dehydration			
	Urine flow	Creatinine clearance	-Δ Urine	Urine flow	Creatinine clearance	-Δ Urine	Urine flow	Creatinine clearance	-Δ Urine	-Δ Plasma
	ml/min	ml/min	C	ml/min	ml/min	C	ml/min	ml/min	C	C
D	1 89	34 5	0 19	3 52	42 0	0 15	0 21	21 6	0 90	0 69
A	1 57	40 8	0 22	5 40	48 8	0 14	0 53	36 8	0 59	0 63
B	1 40	31 3	0 21	2 84	35 2	0 16	0 19	24 2	1 01	0 62
C	1 59	30 6	0 19	4 44	42 2	0 16	0 21	26 9	0 94	0 71

State of Hydration versus Urine Flow—Our earliest observations suggested that in diabetes insipidus, as in the normal animal (15), there is rough correlation between the state of hydration, the rate of glomerular filtration, and the rate of urine formation. Furthermore, the oliguria of dehydration is frequently accompanied by freezing point depressions of urine which are higher than those concurrently found in plasma. These relationships were examined systematically in a series of experiments.

Four experiments are presented in summary form in Table II and are representative of the usual findings. The initial observations were obtained 16 hours after the last meal, while the animals had free access to water. Water was then administered by stomach tube and 1 hour later a second series of observations was made. The animals were fed and drinking water was permitted for the subsequent 4 hours, at which time it was removed. The final series of observations was made 18 hours later. Each series consisted of three experimental periods.

These experiments yield supportive evidence for the existence of a general relationship between hydration, glomerular filtration rate, and urine flow, but

as in the normal dog (15), the relationship is only clearly apparent at the extremes of hydration. There is usually some fall in glomerular filtration rate during dehydration and this may be an important factor in the production of oliguria. It must be emphasized, however, that the oliguria does not involve any great fall in the rate of glomerular filtration but is in part attributable to tubular reabsorption of water as shown by the attainment of moderately high creatinine U/P ratios and the frequent occurrence of urine hypertonicity. However, the capacity of these animals to form a hypertonic urine was considerably less than that of the normal dog with comparable dehydration (*cf* 16), as was their ability to concentrate creatinine. The creatinine U/P ratios were usually below 200 in the animals with diabetes insipidus and we observed a value as high as 300 on only one occasion. Normal animals with comparable dehydration reach creatinine U/P ratios of 500 to 800 (15). Dog B (see

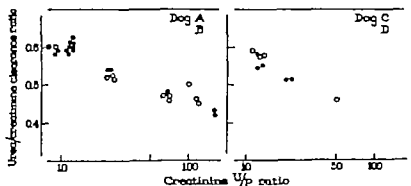


FIG. 1 The relationship between the urea/creatinine clearance ratio and the creatinine U/P ratio in four dogs with diabetes insipidus

Table II) concentrated more extensively than the remaining three animals, and it will be noted that this animal was judged to have the largest quantity of residual supraoptic hypophyseal tissue. A hypertonic urine was not found in the experiment on dog A given in Table II, but was present on other occasions during similar dehydration.

Mechanism of Water Reabsorption during Dehydration—There are relatively few means by which water reabsorption can be compared in diabetes insipidus and in the normal animal in terms of renal tubular mechanisms. There are, however, three phenomena which may be expected to yield some critical information: the minimal creatinine or inulin U/P ratio during water diuresis, the relationship between urea reabsorption and water reabsorption, and the identity of simultaneously observed creatinine and inulin clearances at low urine flows.

It is unusual for the inulin or creatinine U/P ratio to fall significantly below 10 during water diuresis (40 to 50 ml. per kilo) in the normal animal (15) presumably because 85 to 90 per cent of the filtered water is reabsorbed in the proximal portions of the nephron (17-19). The fact that the creatinine or

inulin U/P ratio during maximal water diuresis does not fall below this same range in diabetes insipidus (Fig 1, Table II) indicates that such proximal reabsorption of water is unimpaired. The normal value of the urea/creatinine clearance ratio at the high urine flows is supportive evidence for this belief. When the creatinine U/P ratio is elevated above 10 by an increase in the fraction of water reabsorbed, there is an accompanying increase in urea reabsorption in dogs with diabetes insipidus (Fig 1) which is much the same as in the normal animal (15). Such parallel behavior would be an unlikely finding if water reabsorption in the animal with diabetes insipidus differed qualitatively from that in the normal animal.

The simultaneously determined creatinine and inulin clearances appear to be identical in diabetes insipidus (Table III). This finding is important if creatinine or inulin is to be used in the measurement of glomerular filtration.

TABLE III

A Summary of Experiments Which Compare the Simultaneous Inulin and Creatinine Clearances

Dog	Periods	Urine flow		Inulin U/P ratio		Mean clearance		Creatinine/inulin clearance ratio		
		Mini mum	Maxi mum	Mini mum	Maxi mum	Inulin	Creati nine	Mini mum	Maxi mum	Mean
		<i>ml/min</i>	<i>ml/min</i>			<i>ml/min</i>	<i>ml/min</i>			
B	3	0 117	0 400	72 0	223	25 8	25 8	0 960	1 030	1 002
B	3	0 139	0 161	154 0	186	26 2	25 2	0 950	0 984	0 969
B	5	0 166	0 582	48 4	134	23 7	23 8	0 960	1 055	1 000
A	3	0 235	0 660	60 5	160	39 2	38 1	0 944	0 995	0 975
A	3	0 125	0 197	213 0	329	40 5	40 6	0 980	1 030	1 003

rate. An abnormal type of water reabsorption is also ruled out by these experiments, such as that which we have observed in the surviving kidney when perfused by a pump-lung circuit and which is characterized by the reabsorption of creatinine when the reabsorption of water is great (20).

The Origin of Water Excreted during Dehydration—The mechanism by which animals with diabetes insipidus discard large volumes of water and still maintain circulatory competence and a relatively high glomerular filtration rate was investigated.

The animals were kept in metabolism cages and water intake was measured for a period of 3 days before the control observations. 16 hours after the last meal minimal amounts of sodium bromide or sodium sulfocyanate were injected and 2 hours later the experiment was started by a urine collection period followed by a blood sample. The animals were then weighed and returned to the metabolism cage without food or water for 24 hours. The bladder was emptied, a urine collection period taken, the blood sampled, and the animals reweighed. They were then permitted to satisfy their thirst by giving free access to water for 60 minutes.

A typical experiment² is summarized in Table IV. The urinary loss of water was 0.91 liter and the total weight loss 1.30 kilos. Since no fecal material was passed, it can be assumed that the loss of water by all routes was close to the higher figure. 1.30 kilos is 42 per cent of the volume of extracellular fluid as measured in this animal by the volume of distribution of the bromide ion (11). However, calculations through the change in serum sodium (21) indicate that the contraction of the extracellular fluid compartment was less than half of this value, and it must be concluded that a large portion of the fluid lost was derived from the intracellular compartment. The dehydration may have

TABLE IV

An Experiment Which Examines into the Source of the Water Lost during a 24 Hour Period of Dehydration

Dog C. The water turnover was 3.6, 3.5 and 3.8 liters in the 3 days preceding the experiment. 0.91 liter of urine was excreted ($\text{Na} = 4.5 \text{ mM}$ per liter) during the dehydration period. At the termination of the experiment the animal drank 3.04 liters of water.

	Initial	Final
Body weight kg	12.00	10.71
Extracellular fluid (Br) liters	3.04	2.51*
Serum		
Total solids, gm per cent	8.2	11.04
Sodium, mM/liter	145.3	175.5
Halide, mM/liter	110.6	132.0
$-\Delta \text{ C}$	0.59	0.70
Urine		
Rate, ml/min	1.58	0.15
Sodium, mM/liter	9.6	3.8
Halide mM/liter	8.1	5.2
$-\Delta \text{ C}$	0.18	1.02

* Through sodium.

changed the distribution of some ions but such changes could not be extensive enough to invalidate this interpretation. The change in the total solids of plasma indicates that the plasma volume was contracted to about the same extent as the volume of extracellular fluid. The contraction is apparently small enough and takes place slowly enough so that adjustments in the systemic and renal circulation maintain an adequate filtration pressure in the glomeruli (Table II).

The mechanism by which intracellular water is made available for renal excretion in this type of dehydration is quite clear. On the one hand the essen

² The results in the other experiments were similar to those of the experiment shown in Table IV; the only difference was in the extent of water lost. In terms of body weight this was dog B 0.79 kilo, dog A 0.78 kilo, dog D, 1.20 kilos.

tially complete renal tubular reabsorption of sodium, coupled with the extracellular position of this ion (21), results in the withdrawal of water from the cells as sodium is retained in the body during a period when sodium-free water is discarded by renal action. On the other hand, the complete reabsorption of sodium and hence its retention in the body is facilitated by a reduction in the rate of glomerular filtration (see below), as well as by a deficiency of anti-diuretic hormone (22).

Effect of Saline Ingestion on the Magnitude of Polyuria—The circumstances which surround the increase in the polyuria of diabetes insipidus when the drinking fluid is changed from water to a sodium chloride solution (23, 13) were examined in three of the animals.

Three general experimental routines were used and each type of experiment was performed at least once on each of the three dogs. The animals were studied when at equilibrium with water, with 0.5 per cent NaCl solution, and on return to water (Table V), the minimal period with each drinking fluid being 2 days. They were observed as they proceeded from equilibrium on water to equilibrium on 0.5 per cent saline (Table VI), and as they returned to equilibrium on water again (Table VII). Continuous observations were possible in the last two types of experiments because of the prompt attainment of equilibrium. All experimental periods were terminated by catheterization. Drinking fluid, whether water or 0.5 per cent saline, was always available in excess of the desires of the animals. The animals were maintained in large metabolism cages except during sample collections, and these were performed as expeditiously as possible. This was essential if uncontrolled variables were to be minimized, since the fluid turnover was so large on the saline solution.

Polyuria³ and a greater variability than normal in the volume of extracellular fluid, serum total base or sodium, and glomerular filtration rate are the only outstanding abnormalities which differentiate the water and electrolyte pattern of animals with diabetes insipidus from the normal when drinking water is freely available. A change in the drinking fluid from water to 0.5 per cent saline increased the severity of the polyuria in our experiments, as was expected (Table V). There were in addition a marked elevation of the glomerular filtration rate, and a moderate increase in the volume of extracellular fluid and of plasma and in the concentration of the total base in serum. Potassium analyses were unsatisfactory in our laboratory at the time of these experiments but it was clear that the increase in total base was due to an increase in the concentration of sodium and that potassium was displaced from the body by renal excretion.

A description of the mechanism by which saline ingestion enhances the polyuria of diabetes insipidus appears to require an elucidation of the factors which

³ The polyuria was somewhat less in dogs A and C during this period than that previously observed (Table I) or that observed subsequently. This finding may be attributed to the circumstance that they were being maintained on one-half their usual food intake as a weight-controlling measure (13, 14).

result in the increase in glomerular filtration and the consequences of such an increase on the excretion of water and electrolyte. The sequence of events is shown in Table VI. On ordinary drinking water the animal has a median rate of glomerular filtration and a moderate urine flow which are determined in

TABLE V
Equilibrium States on Water Saline (85 mm per Liter) and on Return to Water

Day	Fluid intake		Serum		Glomerular filtration rate	Urine sodium	Water balance		Diet
	Water	0.5 per cent NaCl	Total base	Total solids			Body weight	Extra cellular fluid†	
	liters/day	liters/day	mm./liter	gm./100 ml	ml./min	mm./liter	kg	liters	
Dog A									
1-5	2 38		154 3	8 21	33 8	12 0	12 1	2 42	½ regular
6§		13 70	158 7	7 39		84 3	13 7	3 50	
7		14 94	160 2	7 36	64 9	85 0	12 9	3 31	
10	2 56		152 8	8 16	34 6	14 5	12 2	2 50	
Dog B									
1-5	4 19		156 7	8 31	33 1	9 3	8 30	2 15	Regular
6§		17 4	165 2	7 60		86 0	9 05	2 86	
7		18 6	164 5	7 51	66 2	85 5	9 10	2 94	
10	4 80		157 2	8 20	35 4	12 6	8 35	2 16	
Dog C									
1-5	2 94		154 3	8 64	31 7	9 6	12 15	2 61	½ regular
6§		12 2	159 9	8 04		87 0	13 10	3 56	
7		11 96	161 1	8 03	56 2	86 2	12 50	3 28	
10	2 95		153 2	8 30	30 8	13 2	12 10	2 65	

* Urine sample taken at termination of each day

† As approximated by sulfocyanate.

‡ Between the 5th and 6th days an 8 hour period was allowed for equilibration with the saline solution. This period was not analyzed.

part, by the degree of hydration (see above) and the urine is essentially free of sodium (period 1). With the ingestion of saline there occurs an expansion in the volume of extracellular fluid and plasma (see accumulated balance, Table VI) through mechanisms not yet elucidated this expansion leads to an increase in glomerular filtration. This in turn produces an increase in urine flow which is not at first accompanied by an equivalent increase in the excretion of sodium the concentration of urinary sodium being below that of the ingested saline. Loss of water, therefore, proceeds without a comparable loss of sodium and an

increase in the concentration of extracellular electrolyte occurs before there is an opportunity to restore the volume of the extracellular compartment to its initial value. The thirst mechanism is stimulated by this increase in the concentration of extracellular electrolyte (24) and the animal takes a second drink of saline (periods 2 to 3). The ingested fluid, being hypotonic, dilutes extracellular electrolyte and thirst is temporarily satisfied. However, the ingested saline further expands the extracellular fluid and plasma volume and leads to a further increase in the filtration rate, this cycle proceeding until the filtration rate of sodium is in excess of the tubular reabsorptive capacity⁴ in the proximal segment. Sodium is now excreted in significant amounts, but at a urine concen-

TABLE VI

The Process of Attaining Equilibrium When an Animal with Diabetes Insipidus Is Placed on Saline (85 mM per Liter)

Period	Time	Intake		Output		Accumulated balance		Glomerular filtration	Plasma		Urine	
		Water	Sodium	Water	Sodium	Water	Sodium		Total base	Total solids	Rate	Sodium
	hrs.. min	ml	mM	ml	mM	ml	mM	ml / min	mM/liter	gm / 100 ml	ml / min	mM/liter
1	00 1 05			111				29.8	155.2	9.05	1.71	6.5
Placed on saline solution 85.5 mM/liter												
2	2 10	330	28.2	170	2.7	160	25.5		158.5	8.67	2.62	16.0
3	3 00	320	27.4	217	7.4	263	45.5	38.2	159.9	8.14	4.34	34.0
4	4 08	540	46.2	517	16.0	286	75.7		160.7	7.79	7.60	30.8
5	5 20	1090	93.2	635	27.4	741	141.5	57.7	160.5	7.78	8.82	43.2
6	6 47	730	62.4	956	75.0	515	128.9		161.5	7.91	11.00	78.4
7	7 45	1080	92.4	980	81.8	615	139.5	61.2	162.0	7.91	16.9	83.5
8	8 51	890	76.0	960	81.4	545	134.1	62.3	161.8	7.88	14.6	84.8

tration still below that of the ingested fluid (periods 3 to 5), and hence concentration of extracellular electrolyte again occurs as water is discarded in excess of sodium.

After several of these episodes glomerular filtration is increased to such an extent that the filtration of sodium considerably exceeds the reabsorptive capacity of the proximal segment, and the sodium concentration of the urine

⁴ Reabsorptive capacity is used in a very broad sense throughout this paper and is not the equivalent of T_m as used to describe the transfer maxima of organic substances such as glucose (25). There is as yet no simple means of quantitating the capacity of the system responsible for the transfer of sodium under any given set of circumstances. The ability of the normal dog to maintain a normal electrolyte balance despite wide fluctuations in filtration rate, the result of dietary régime, is an indication of the functional adaptability of this system.

attains that of the ingested fluid. As long as the animal continues to drink the saline solution at will, it remains in a more or less steady state with respect to both water and sodium (periods 6 to 8). A true steady state is not established, however, since the drinking may be episodic rather than continuous and there is a lag between the drinking of saline and its absorption. Following each drink of saline a period of high urine flow produces a progressive contraction of extracellular fluid and plasma volume, which finds renal expression in a lowered fil

TABLE VII

The Process of Attaining Equilibrium When an Animal Equilibrated with Saline (85 mEq per Liter) Is Returned to Water

Period	Time	Intake		Output		Accumulated deficit		Glo- merular filtra- tion	Plasma		Urine	
		Water	Sodium	Water	Sodium	Water	Sodium		Total base	Total solids	Rate	Sodium
	hrs min.	ml.	mEq	ml	mEq	ml.	mEq	ml / min	mEq/liter	gm./ 100 ml.	ml / min.	mEq/liter
	00											
1	10			140	10.9	140	10.9	63.2	158.6	8.45	14.0	78
2	20			135	10.9	275	21.8	62.4			13.5	81
3	1 00			372	29.4	647	51.2				9.3	79
4	1 10			62	4.8	709	56.0	43.1	164.9	9.04	6.2	77
5	1 20			43	3.3	752	59.3	37.7			4.3	76
6	1 36			43	2.8	795	62.1	34.2	179.5	9.53	2.5	66
7	1 40	660				135	62.1					
8	2 10			150	8.7	285	70.8				4.41	58
9	2 20			59	1.9	344	72.7	48.9	152.3	9.07	5.9	33
10	2 31 5			49	1.1	393	73.8	43.4			4.26	22
11	3 20			110	1.3	503	75.1				2.27	12
12	4 19			84	0.6	587	75.7				1.42	6.0
13	4 32			12.8	0.1	600	75.8	32.0	164.0	8.93	1.03	6.9
14	4 42			8.8	0.1	609	75.9	32.0			0.88	6.9
15	4 50	520				89	75.9					
16	5 30			115	3.0	204	78.9				2.60	26

tration rate and decreased sodium excretion, the excretion of water in excess of sodium produces an increased concentration of electrolyte in the extracellular fluid and the animal is stimulated to drink, thus initiating the following oscillation. The experiment given in Table VII was selected to illustrate this portion of the cycle as well as the reversion to normal through the drinking of water. It shows the contraction of extracellular fluid and its sequelae particularly well since the animal refrained from drinking for a considerable period of time.

When the animal drinks water a prompt reversion to the normal state occurs. The mechanism of the reversal is illustrated by the experiment presented in Table VII. The initial effect of the ingested water is qualitatively the same

as if saline were taken, the increase in filtration rate being somewhat less since the water is distributed in a larger volume than is saline. However, the increase in filtration rate permits the continued excretion of sodium, creating a sodium deficit since the fluid absorbed is sodium-free. The fluid exchange returns approximately to the pre-saline level when extracellular fluid and plasma volume and consequently the filtration rate have been reduced to normal, and when sufficient sodium has been excreted so that its concentration in the extracellular fluid compartment is in the normal range. This usually takes place in a period of 4 to 6 hours.

DISCUSSION

It is accepted that the polyuria of diabetes insipidus is attributable to a deficiency of the antidiuretic hormone of the posterior pituitary gland. Owing to this deficiency the active tubular reabsorption of water in the distal segment or distal portions of the nephron is quantitatively unimportant under conditions of ordinary hydration so that a markedly hypotonic urine is formed. Nevertheless, the renal response to dehydration is similar to the response of the normal animal. It may be that the chronic polyuria does not exclude the presence of sufficient posterior pituitary tissue with which to form the antidiuretic hormone in significant amounts during dehydration. Alternatively, the nephron may be able to reabsorb water against an osmotic gradient in the complete absence of the antidiuretic principle. The anatomical and functional data collected from dogs C and D incline us to the latter view according to which the antidiuretic hormone facilitates or accelerates the hypertonic reabsorption of water but is not essential for the process.

In either of these views dehydration in both normal and diabetes insipidus animals would impose upon the distal segment the conditions favoring the formation of a highly concentrated urine. The complete reabsorption of electrolyte, particularly of sodium, high in the proximal segment (19) would be the first essential for this operation. A reduced filtration rate would facilitate this process as well as the early attainment of osmotic equilibrium between tubular contents and renal interstitial fluid through the passive reabsorption of water. Such reabsorption of water may be expected to take place in the proximal segment, Henle's loop, and possibly in the distal segment and to proceed in response to the force established by the active absorption of osmotically active material in the proximal segment (3). Under these circumstances only small quantities of fluid are delivered to the distal segment and oliguria may result from this mechanism alone, in the absence of any active force applied to water itself. Data obtained from a normal dog which emphasize this concept are presented in Fig 2. Thermodynamically no work is directly performed on water in this series of observations until the urine flow is below 0.4 ml per minute, at which time the freezing point depression of urine is equal to that of plasma.

It seems likely, from these considerations, that the quantitative relationship between glomerular filtration and proximal tubular reabsorption, particularly with respect to the electrolytes, is an important determinant of the rate of water excretion in the normal animal. The control of (distal) reabsorption of water by the antidiuretic hormone in the normal animal is a device added to the above mechanism contributing both a more rapid and a further conservation of water by an active process permitting the elaboration of a highly hypertonic urine

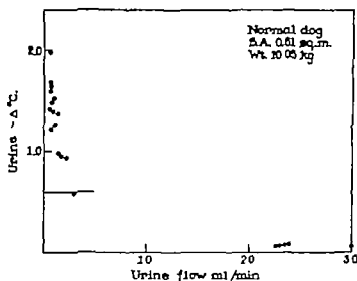


FIG. 2 The relation between urine flow and the osmotic pressure of the urine in a normal dog on a low protein diet. Each point represents a single experimental observation. S.A. indicates surface area. The line drawn horizontally at $-\Delta = 0.6^\circ \text{C}$ represents the mean of a series of plasma observations obtained during moderate dehydration. These relationships are not fixed in any animal but will vary depending upon variations in glomerular filtration rate, food and salt intake. Consequently there will be a variation in the lower limit of the urine flow which thermodynamically does not require the application of work on the water in the system.

The importance of such quantitative relationships in the proximal portions of the nephron in the overall determination of water excretion is fortified by the experiments which examine the enhancement of polyuria in diabetes insipidus when saline is ingested. Two fundamental derangements in electrolyte-water balance appear to be involved in this phenomenon, both being attributable to a deficiency of antidiuretic hormone. They are an increase in the reabsorptive capacity for sodium proximally in the nephron and an impairment in the active reabsorption of water distally (22). When saline is ingested both derangements, together with the normal operation of the thirst mechanism, favor the progressive localization of sodium in the body with a consequent increase in the volume of extracellular fluid and plasma. Circulatory adjustments result in a progressive increase in the rate of glomerular filtration and it appears that this

increase is the immediate cause of the increased polyuria as well as the circumstance which permits the ultimate attainment of sodium balance

The mechanism which is responsible for the increase in urine flow appears to lie in the circumstances which surround the reabsorption of water in the proximal portions of the nephron. Normally, we suppose the reabsorption of solute proceeds more rapidly than the passive reabsorption of water, so that tubular urine is hypotonic as it enters the distal segment. Otherwise, it is unlikely that a hypotonic urine would be formed in those conditions in which the active reabsorption of water distally is minimal, *i. e.*, in diabetes insipidus or during water diuresis in the normal animal. An increase in glomerular filtration rate, by diminishing the time available for the equilibration of the tubular urine will result, under these conditions, in a smaller percentage reduction in the diffusion gradient of water and hence will deliver fluid to the distal segment at a higher rate.⁵ As the progressive elevation of filtration continues and the reabsorption of electrolyte becomes less complete there is added to this increased flow factor a reduction in the diffusion gradient of water itself, in consequence of the presence of unabsorbed sodium. This contributes to the increase in the rate of delivery of fluid to the distal segment. Some reabsorption of water presumably continues to occur in the distal segment at all times owing to the incomplete dissipation of the diffusion gradient of water. However, without the antidiuretic hormone to facilitate or accelerate distal reabsorption, this process remains quantitatively unimportant in reducing the polyuria.

It may be suggested, as a logical extension of the discussion, that the urine formed in diabetes insipidus during normal hydration approaches in character that which, under the same circumstances, would be delivered to the distal segment in the normal animal. These experiments are then examples of how variations in the quantitative balance between glomerular filtration and proximal tubule reabsorption can vary the amount and character of the fluid presented to the distal segment, thereby contributing directly to the regulation of the volume and composition of the urine (18) and indirectly to that of the body fluids.

SUMMARY

Water and electrolyte excretion has been studied in a series of dogs with diabetes insipidus, in which the extent of neurological damage was subsequently determined. The animals were studied before and after the introduction of variables which produce marked changes in the state of hydration,—administration and restriction of water and the substitution of 0.5 per cent sodium chloride for it as a drinking fluid. Observations were made on those factors,

⁵ More generally, this would not be expected to obtain under conditions in which the initial state involved an extensive reduction in glomerular filtration rate, or, in which the increase in glomerular filtration was accompanied by a comparable increase in the ability of the tubules to reabsorb sodium.

both general and renal, which appear to be important in determining the excretion of water and electrolyte, or which may be expected to yield information on the mechanisms by which the regulation of such excretion is achieved. These are the volume of extracellular fluid and plasma and the concentration of the contained electrolyte, glomerular filtration rate, and the excretion of electrolyte, urea, and water itself, as well as the tonicity of the urine.

CONCLUSIONS

1 Changes in the renal excretion of water, in response to extensive changes in hydration, appear to proceed in the dog with diabetes insipidus in a fashion which is qualitatively similar to that in the normal animal. The oliguria produced by dehydration is frequently characterized by a hypertonic urine and requires the active tubular reabsorption of water. This type of oliguria does not appear to require the presence of the antidiuretic hormone.

2 Extensive dehydration in dogs with diabetes insipidus, when due to the renal loss of water, is not accompanied by circulatory collapse or an extensive fall in glomerular filtration rate. This circumstance is related to the maintenance of an adequate volume of extracellular fluid in consequence of the complete tubular reabsorption of filtered sodium and the extracellular position of this ion.

3 The enhancement of the polyuria of diabetes insipidus which results from the ingestion of saline is due to an exaggeration of the deficiencies in renal function caused by an inadequate supply of the antidiuretic hormone. These are an increased capacity for the reabsorption of sodium proximally and a diminution in the active reabsorption of water distally. The saline régime, under these circumstances, produces a localization of extra sodium in the body, an increase in the volume of extracellular fluid and plasma, and an increase in glomerular filtration rate. The increase in glomerular filtration rate is the fundamental change which both produces the increase in urine flow and permits the establishment of sodium balance.

4 The same variables appear to be responsible for the low urine flow of dehydration and the excessively high flow when saline is used as a drinking fluid in diabetes insipidus. They arise from the circumstances which surround the proximal reabsorption of water and electrolyte and the quantitative relationship between these and the rate of glomerular filtration which together determine the volume of fluid delivered to the distal segment.

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THE CONTROL OF THE RENAL EXCRETION OF WATER*

II. THE RATE OF LIBERATION OF THE POSTERIOR PITUITARY ANTIDIURETIC HORMONE IN THE DOG

By JAMES A. SHANNON M.D

(From the Departments of Physiology and Medicine New York University College of Medicine and the Research Service Third (New York University) Medical Division Welfare Hospital New York)

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These experiments were performed for two purposes. The first was to define the normal rate of liberation of the antidiuretic hormone. The action of the hormone on the renal tubular reabsorption of water is fairly clear, at least in its qualitative aspects. Its normal participation, with the suprarenal cortical hormone, in control of the renal tubular reabsorption of sodium (1-3) is less certain because of the excessive amounts of antidiuretic hormone commonly used to demonstrate such an action. A decision on the function of the antidiuretic hormone and its relation to other variables in the two systems cannot be arrived at until its normal rate of liberation is defined.

The second purpose was the clarification of those relationships within the nephron which appear to be important in determining the volume as well as the composition of the final urine. There is normally a rather precise balance between the glomerular filtration rate and tubular reabsorptive capacity (proximal) in the individual nephra which permits the kidney to function as if the operations of filtration and reabsorption were taking place in a single nephron (4, 5). This balance is particularly important in the case of electrolyte if, as seems likely, the reabsorption of osmotically active material in the proximal portions of the nephron (6) indirectly provides the force for the reabsorption of a large portion of the filtered water (5, 7). A disturbance of the normal relationship, such as an increase in glomerular filtration without a comparable increase in the reabsorptive capacity of the tubules, establishes conditions which favor an increase in the rate of urine flow. The converse is also to be expected (8).

EXPERIMENTAL

Material—Seven female dogs were used. Four dogs had permanent diabetes insipidus and their state has been previously described (8) the remainder were normal. The following data (May 6 to 13 1940) on the weight and urine flow of the diabetes

* This investigation has been aided by a grant from the Daxian Foundation for Medical Research

insipidus dogs are typical of the period covered by these experiments Dog A, 11.65 kilos, 3.45 liters per day, dog B, 7.25 kilos, 4.15 liters per day, dog D, 8.8 kilos, 4.21 liters per day, dog C, 11.3 kilos, 3.34 liters per day The slight reduction of the polyuria, as compared to that of the initial observations may be attributed, if significant, to quantitative changes in the diet (9, 10) rather than to an essential change in the fundamental deficiency in the animals The normal dogs weighed between 12 and 15 kilos

Methods—The method of administration of a hormone is of particular importance if the collected data are to be used to define its normal rate of liberation The procedure must mimic the normal process as far as is experimentally possible Administration of the hormone in our experiments was by a slow infusion into the marginal ear vein of the dogs at a rate of 5.0 or 10.0 ml of fluid per hour The infusion arrangements were as follows The shaft of a 20 gauge, short beveled needle was used as a cannula and was connected to an infusion pump by small caliber, light weight rubber tubing The tubing was clipped to the dog's head and the cannula was attached to the ear by a paper clip, with the edge of the ear supported by a small piece of celluloid Both ears were then folded and taped together for added stability Such arrangements allow the animals considerable freedom and permit prolonged observations without requiring the use of restraints The shortest time which elapsed between the beginning of a hormone infusion and the beginning of an experimental observation was 25 minutes, frequently a longer preliminary adjustment period was permitted, particularly at the low rates of hormone administration

Three general types of experiments were performed, some were less extensive than those shown in Tables I to III in that fewer variables were examined serially The preparation of the animals was the same for all experiments unless stated to the contrary They were permitted water up to an hour before the beginning of the experiment, which was performed 16 to 20 hours after the last meal Creatinine for the measurement of glomerular filtration rate was injected subcutaneously or included in the infusion fluid The general experimental routine, the preparation of samples for analysis, and the chemical methods were the same as those previously used by us (8) A single preparation of pituitary liquid (U.S.P., Armour) was used throughout Bioassay, at the beginning and termination of the study, showed it to contain 10 pressor units per ml of solution¹ The hormone infusions were prepared by dilution with physiological saline solution acidified with hydrochloric acid to a pH of 5.5–6.0 Such solutions of hormone are quite stable However, fresh dilutions of hormone were made each 60 to 90 minutes

RESULTS

Antidiuresis—The following observations on dogs with diabetes insipidus relate to the antidiuresis produced by the constant intravenous infusion of the antidiuretic hormone Some of the salient features of this portion of the data are summarized in Fig 1 The experiments were similar in design to that of Table I and the early portion of the experiment of Table II

¹ We wish to thank Dr E. M. K. Geiling for the bioassays at the termination of the study

1 Administration of the antidiuretic hormone was not accompanied by a change in the rate of glomerular filtration in the range of hormone administration used (1.0 to 350 millunits per hour). All changes in urine flow were there for the result of changes in the reabsorption of water.

2 There was considerable variation in the degree of antidiuresis produced by a given level of hormone administration in the different animals and in each animal at different times. This was true however the antidiuresis is evaluated,

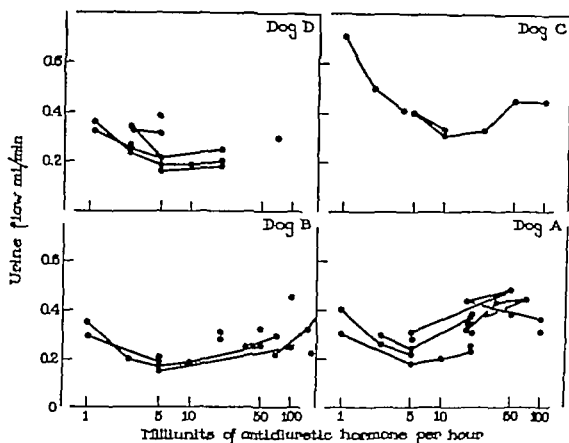


FIG 1 The urine flow in four dogs with diabetes insipidus at various rates of administration of the antidiuretic hormone. The data were collected in experiments similar in design to that of Table I and the first portion of that of Table II. Each point is the mean of two or three consecutive periods. The lines connect observations obtained serially; the unconnected points are from experiments in which a single rate of hormone administration was examined.

i.e., as absolute or percentile reductions in water excretion or as absolute rates of water excretion.

3 Graded antidiuresis with graded hormone administration was consistently observed only in the range of 1.0 to 5.0 millunits per hour.

4 When a single experiment included a series of progressively increasing rates of hormone administration maximal antidiuresis was usually reached at 5.0 millunits of hormone per hour. There was never a further increase in the antidiuresis when the hormone was raised from 20 millunits to some higher

value Under the latter condition the urine flow remained the same or increased with the increase in the rate of hormone administration

5 The minimal urine flows during hormone antidiuresis were frequently higher than those sometimes observed in the same animals after simple dehydration for 18 to 24 hours However, in hormone antidiuresis the urine was consistently more hypertonic than in the oliguria of simple dehydration (Compare Fig 1 with Table II, in the preceding paper)

TABLE I

An Experiment on a Dog with Diabetes Insipidus Which Examines into Some Effects of the Administration of the Antidiuretic Hormone by Constant Intravenous Infusion

This experiment is typical in that it shows no significant change in glomerular filtration rate, graded antidiuresis in the lower range of hormone administration, and an increase in electrolyte excretion which is related to the hormone administration

Dog D, weight 8.8 kilos Creatinine was incorporated in the infusion fluids, its plasma concentration varied from 5.31 to 5.67 mg per cent, the serum sodium was 149.0, the chloride 114.2 mEq per liter

Period	Concurrent time	Urine flow	Creatinine U/P ratio	Creatinine clearance	Urine Na	Urine Cl	Na excretion	Cl excretion
	min	ml/min		ml/min	mEq/liter	mEq/liter	mEq/min	mEq/min
1	0-21	2.10	16.3	34.2	6	8	0.013	0.017
2	-40	2.42	14.7	35.6	7	8	0.017	0.019
	43	Pituitrin infusion 0.001 unit per hr						
3	75-91	0.33	106	35.0	63	60	0.021	0.020
4	-110	0.32	104	33.2	68	62	0.022	0.020
	112	Pituitrin infusion 0.0025 unit per hr						
5	141-161	0.24	141	33.8	112	104	0.027	0.025
6	-180	0.25	140	35.0	118	107	0.030	0.027
	183	Pituitrin infusion 0.005 unit per hr						
7	210-230	0.21	165	34.7	161	147	0.034	0.031
8	-249	0.21	172	36.1	157	145	0.033	0.030
	254	Pituitrin infusion 0.020 unit per hr						
9	281-303	0.26	137	35.6	169	152	0.044	0.040
10	-325	0.25	137	34.5	172	154	0.043	0.039

6 Urine flows at true oliguric levels (i.e., 0.1 ml per minute or less in a 10.0 kilo dog) were only obtained by the combination of moderate to severe dehydration and hormone administration This régime produced creatinine U/P ratios comparable to those commonly observed with severe dehydration in the normal dog (11)

7 The administration of 40 to 50 ml of water per kilo elevated the range of hormone administration which produced graded antidiuresis and diminished the degree of antidiuresis at a constant rate of hormone administration

These observations define the quantitative relationship between hormone ad-

ministration and antidiuresis in the dog with diabetes insipidus, in so far as a definition can be made in terms of the overall effect, *i. e.*, rate of urine flow. Of equal importance is the support which they give to the viewpoint that the antidiuretic hormone is only one of a series of variables which are concerned in the production of variations in the rate of urine formation.

Sodium and Chloride Excretion—Observations on the influence of the antidiuretic hormone on the excretion of electrolyte were limited to sodium and chloride. The experiments were similar in design to the one shown in Table I and the first portion of that in Table II and were, for the most part, the experiments from which Fig. 1 was constructed. There were some additional experiments on dogs B and C which differ somewhat from the remainder in that minor variations in the water balance of the animals were induced. Both ions were studied in the majority of cases but in some only chloride was observed. Variations in the excretion of sodium in these experiments were accompanied by equivalent variations in the excretion of chloride. For this reason and because the chloride data are more extensive we have chosen them for presentation (Fig. 2).

An outstanding characteristic of these data is the great variability in the effect of the hormone on electrolyte excretion. There was a significant increase in the excretion of sodium and chloride at low rates of hormone administration (*i. e.* 20 millunits per hour or less) in three dogs while in the fourth (dog D) no systematic and reproducible effect, which could be attributed to the action of the hormone, was observed. The higher ranges of hormone administration were not examined in the latter animal. The increase in excretion observed in the other three animals represents an equivalent depression in renal tubular reabsorption since glomerular filtration rate is not affected in this range of hormone administration. It should be noted that small percentile depressions in the reabsorptive process will result in large percentile and large absolute increases in the excretion rate of sodium and chloride because of the relationship that normally exists between their filtration, reabsorption and excretion. The variability in the excretion data may result in part from this circumstance.

The effect of the hormone on sodium and chloride excretion is great in dogs A and C when considered in terms of the daily sodium and chloride balance. An increase of 0.01 mM per minute in the excretion of chloride in these animals would result in an additional loss of 14.4 mM in a 24 hour period or about 5 per cent of their total chloride content. However this rate of hormone administration did not affect sodium or chloride excretion as greatly when the animals were moderately dehydrated and this was also the case for normal animals under similar conditions. The latter finding is perhaps correlated with the lowered rate of glomerular filtration (and hence filtration of electrolyte) commonly observed under these conditions (8).

The depression of electrolyte reabsorption frequently produces a higher elec

trolyte concentration in the urine during hormone antidiuresis in the diabetes insipidus animal than is usually observed during oliguria at a comparable urine flow in normal dogs and may operate to limit the degree of antidiuresis which the hormone can produce in a normally hydrated animal with diabetes insipidus (8) The increase in the excretion of electrolyte which may result from an increase in hormone administration is frequently accompanied by an increase in

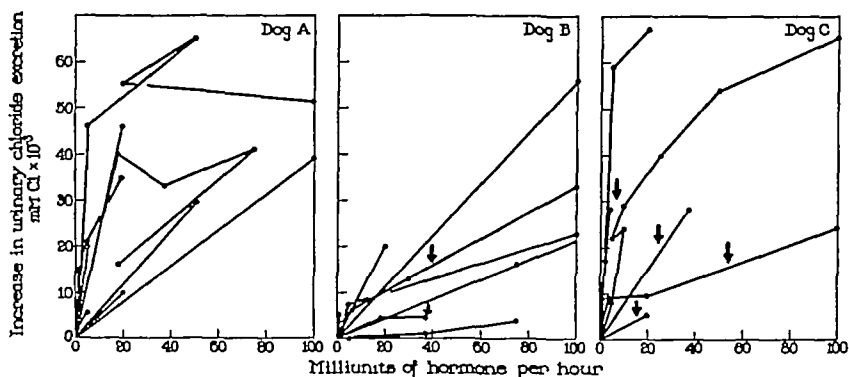


FIG 2 The increase in chloride excretion in dogs with diabetes insipidus at different rates of antidiuretic hormone administration The data were collected in experiments similar in design to that of Table I and of the first portion of Table II Each point is the mean of two or three consecutive periods, the lines connect observations obtained serially Since all experiments have an initial control period the line to the initial observation must of necessity start at the intersection of the coordinates The data from dog A are from the same experiments that were used in the construction of Fig 1, and in the case of dogs B and C additional experiments have been included In two experiments on dog B water had been withdrawn for a period of 4 hours (arrows), in four experiments on dog C 40 ml per kilo of water had been administered 3 hours prior to beginning (arrows) Neither of these procedures was expected to influence the data nor do they appear to have done so Electrolyte excretion was measured in six experiments performed on dog D, and there was no systematic effect on chloride excretion (see text, page 391)

the rate of urine formation The increase in the urine flow appears to be causally related to the electrolyte effect since it occurs only when there is a significant increase in the excretion of electrolyte at the higher rate of hormone administration

The Effect of Water Administration—The effect of water administration on hormone antidiuresis was studied in a series of experiments similar in design to that of Table II There was an invariable increase in urine flow during the 1st hour after the administration of water, which at times reached values above 10 ml per minute (Fig 3) The experimental periods were too long to reveal

the true rate of increase in the urine flow after the water but, in general, it appears to follow the same time course as water diuresis in the normal animal (12). When observed during the 2nd hour the urine flow was usually at a lower value but was above the control rate. There was no close correlation between the extent of the change in urine flow and the rate of hormone administration.

TABLE II

An Experiment on a Dog with Diabetes Insipidus Which Examines into the Effect of the Administration of Water during a Continuous Infusion of the Antidiuretic Hormone

The terminal portion of the experiment demonstrates the rapid lessening in antidiuresis when the hormone infusion is stopped

Dog A, weight 11.6 kilos. Plasma creatinine varied from 9.78 to 13.86 mg per cent the initial serum total base was 159.3 mM, the chloride 110.7 mM per liter

Period	Total concurrent time	Urine flow	Creatinine clearance	Urine Na	Urine Cl	Urine -Δ
	min	ml./min	ml./min	mm./liter	mm./liter	°C
1	0-15	1.86	26.3	11	13	0.18
2	-34	1.84	25.6	12	14	0.18
	45	Pituitrin infusion started 0.020 unit per hr				
3	77-94	0.27	24.6	178	173	1.34
4	-114	0.30	26.3	162	161	1.42
	115	50 ml. per kg. of water by stomach tube				
5*	114-130	0.59	—	—	—	—
6*	-150	0.83	—	—	—	—
7	-165	1.07	35.0	150	119	0.78
8	-181	1.13	38.0	141	113	0.79
9	-202	0.86	37.6	138	106	0.81
10	-222	0.52	35.0	142	111	1.07
11	-241	0.43	38.7	138	99	1.03
12	-262	0.51	35.0	144	110	0.91
13	-284	0.43	38.8	141	100	0.99
		Pituitrin infusion stopped				
14	284-300	0.42	—	—	—	—
15*	-316	1.61	—	—	—	—
16	-334	2.38	—	—	3	—
17	-345	2.15	—	—	2	0.07

* Significant dead space error due to increase in urine flow during periods.

The data indicate, as shown in Table II, that there was an increase in glomerular filtration rate during the 1st hour which was sustained throughout the 2nd except in one experiment on dog A (indicated by an arrow in Fig. 3). Electrolyte excretion also increased consistently after the water, presumably due to the increase in glomerular filtration. However, the concentrations of sodium and chloride and the freezing point depressions of the urines were not regularly sufficient for either to be the immediate cause of the increase in water excretion,

it being unusual for urinary sodium to be in excess of 200 mM per liter and freezing point depressions to be in excess of 1.2°C

The increase in the glomerular filtration rate which commonly follows the administration of a large amount of water is undoubtedly an important factor in producing the increase in urine flow. This effect may be expected to be an exaggeration of that which occurs in the normal animal since, owing to the hormone administration, a major portion of the absorbed water is retained. The mechanism by which such a factor may be expected to operate has been discussed at length in the accompanying paper (8). However, the absence of an

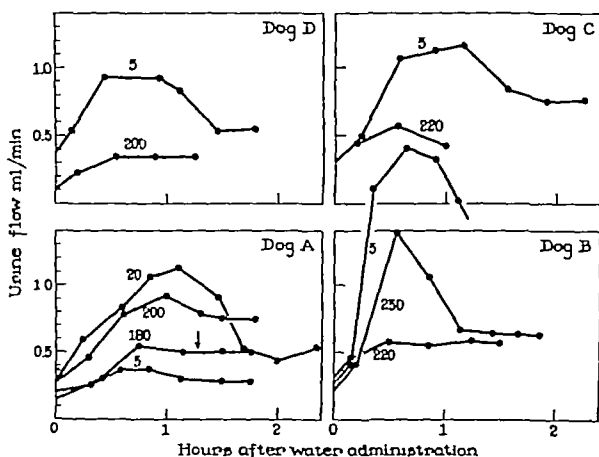


FIG. 3 The influence of the administration of water on the urine flow of dogs with diabetes insipidus during a constant intravenous infusion of the antidiuretic hormone. These experiments are similar in design to that of Table II. The rate of hormone administration in millunits per hour is given by the number above each curve. The points are placed at the midperiod of the urine collection periods.

increase in filtration rate in one experiment and the absence of a close correlation between the increase in filtration and increase in urine flow indicate that other factors are concerned in the phenomenon. It does not seem likely that a change in the dynamics of renal interstitial fluid movement resulting from an expansion in plasma volume or a dilution of the plasma is important since these changes would be expected to be small (5, 13). Nor does the increase in urine flow appear to be conditioned to any extent by a decrease in the reactivity of the distal tubule cells to the antidiuretic hormone since the response is still apparent at high rates of hormone administration (Fig. 3). It is necessary to suppose that the phenomenon is due to a combination of factors which defy exposition at the moment.

Whatever its mechanism, the demonstration of the phenomenon is important

It has been frequently stated that the dog with diabetes insipidus is particularly sensitive to the antidiuretic hormone as compared to the normal animal. Our results suggest that the difference between the two may be due simply to the method of examination and that the apparent insensitivity of the normal animal to the hormone results from the water which must be administered before making the antidiuretic test.

TABLE III

An Experiment on a Normal Dog Demonstrating the Effect of the Antidiuretic Hormone during Water Diuresis

Dog E, weight 14.1 kilos. Creatinine concentration in the plasma varied from 3.5 to 6.3 mg per cent.

Period	Time	Urine flow	Creatinine clearance	Urine Cl	Urine -Δ
	hrs min	ml/min	ml/min	mm/100 ml	°C
	0	50 ml water per kg by stomach tube			
1	1 07-1 17	10 00	86.4	13	0 26
2	-1 29	9 61	86.8	13	0 27
	1 43	Pituitrin infusion 0.005 unit per hr excreted water replaced			
3	2 20-2 30	1 52	86.3	129	1 64
4	-2 40	1 25	80.0	129	1 86
	2 45	Pituitrin infusion 0.020 unit per hr			
5	3 15-3 30	0 53	78.3	185	3 27
6	-3 45	0 58	76.0	185	3 17
	3 47	Pituitrin infusion off			
7	3 45-4 03	0 71	—	179	2 82
8	-4 16	1 84	—	86	1 17
9	-4 31	4 86	77.3	11	0 36
10	-4 47	7 07	78.4	6	0 23
11	-5 02	6 45	76.3	7	0 22
12	-5 34	3 97	—	16	0 37
13	-5 51	0 96	—	70	1 28
14	-6 08	0 65	73.6	108	1 77
15	-6 26	0 40	61.2	116	2 24
16	-6 58	0 37	62.8	110	2 51
17	-7 27	0 37	62.0	105	2 53

Supporting evidence for this view was obtained in experiments performed on normal dogs. Water was administered (40 or 50 ml. per kilo) and a series of pituitrin infusions given at the height of water diuresis. Diuresis was then permitted to resume and continuous observations were made until the urine flow returned to the oliguric level. The water turnover in such experiments is large and it is difficult to reproduce conditions closely. However, the experiment of Table III may be taken as fairly typical except that the freezing point depressions at all urine flows in this experiment are somewhat higher than usually

obtain Antidiuresis with 10 to 50 milliunits of hormone per hour was usually moderate and the urines had higher electrolyte concentrations and greater freezing point depressions than the urines at comparable flows in the absence of exogenous hormone At a higher rate, 20 milliunits of hormone per hour, abnormally large freezing point depressions were commonly observed and truly oliguric levels of urine flow never obtained Subsequent to the return of diuresis, the urine flow reapproached the oliguric level only when the glomerular filtration rate had become depressed These experiments demonstrate directly that the antidiuretic hormone has a pronounced physiological effect in the normal animal at low rates of administration (*i e*, 10 to 50 milliunits per hour),

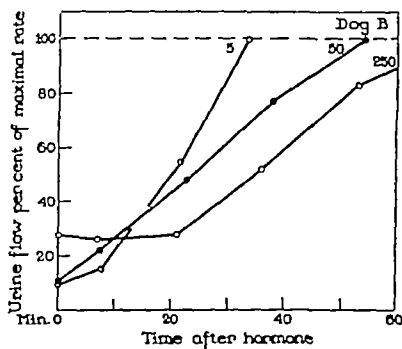


FIG 4 The falling off of antidiuretic activity in a dog with diabetes insipidus, following the cessation of a constant intravenous infusion of the antidiuretic hormone The rate of hormone administration, in milliunits per hour, is given by the number above each curve The time of attaining the maximal urine flow after the cessation of the hormone infusion has been taken as the time which must elapse for the complete dissipation of the antidiuretic hormone in the body The urine flows have been expressed as the percentages of the maximal flow in each experiment and are plotted at the midpoint of the collection periods

and offer an explanation for the apparent insensitivity of the normal animal to this hormone

The effect of a low rate of hormone administration (5 milliunits per hour) was examined during spontaneous antidiuresis in the normal dog There was an invariable reduction in the rate of urine flow unless the animals had been previously dehydrated It can be concluded that normal antidiuresis in the dog does not result from a rate of hormone liberation which is in excess of the amount which produces graded antidiuresis

Falling Off in Antidiuretic Activity—The return to a high urine flow is quite rapid in the dogs with diabetes insipidus when a constant intravenous infusion of the antidiuretic hormone is stopped The lessening in activity has been examined in experiments specifically designed for such a study, and in others by

making use of the terminal portions of an experiment as in Table II. Water had been administered in some experiments but not in the majority. In all, the hormone was administered for a period of at least 1 hour. A series of typical recovery curves is given in Fig. 4. The data of other experiments on dog B and on the other animals are in keeping with the salient features of this figure. Return of the polyuria, or the lessening in antidiuretic activity, is quite rapid in the lower range of hormone administration (i.e., 1.0 to 5.0 milliunits per hour) being complete in less than 30 minutes. The time required for complete recovery increases progressively as the rate of hormone administration is increased.

The peak of water diuresis in a normal dog is reached about 50 minutes after the administration of water (13). The interval must include the time required for the passage of water into the intestine for some of its absorption or for the entrance of electrolyte in the gastrointestinal tract, for the depression in posterior pituitary activity, and for the falling off in the activity of the peripherally placed antidiuretic principle. The time of this last at rates of hormone administration of 5 milliunits of hormone per hour is sufficiently short to be contained within the 50 minute period.

DISCUSSION

The normal rate of liberation of the antidiuretic hormone in a 10 to 15 kilo dog appears to be in the order of magnitude of 1.0 to 5.0 milliunits per hour. It is only in this range that graded antidiuresis is consistently obtained when the hormone is infused intravenously into a normally hydrated dog with diabetes insipidus, and minimal urine flows were usually obtained at its upper limit. The applicability of these data to the normal animal seems justified by the following considerations. The administration of water to an animal with diabetes insipidus reduces the antidiuresis at all levels of hormone administration so that the animal reacts to the hormone in much the same manner as do normal animals during water diuresis. Experiments on the normal dog directly demonstrate that the above range of hormone administration is physiologically effective and that antidiuresis is normally accompanied by the endogenous liberation of hormone at a rate below that which will produce maximal hormone antidiuresis.

The lack of peripheral storage of the antidiuretic hormone and its prompt destruction are of physiological importance. Otherwise, the onset of water diuresis would be delayed and the administration of water would represent a serious physiological hazard. Actually, the rate of destruction is too rapid at physiological rates of administration (i.e., 5.0 milliunits or less) for the lessening of antidiuretic activity to dominate the normal curve of water diuresis. The presence of other determining factors is emphasized by the experiments in which water administered during a constant intravenous infusion of the hormone pro-

duced a prompt increase in the urine flow of dogs with diabetes insipidus. It is safe to conclude that the curve of water diuresis in the normal animal, and perhaps the magnitude of the resulting polyuria, are due to the combination of the latter factors (Fig 3) as well as to the peripheral lessening in antidiuretic activity (Fig 4).

The demonstration of a significant depression in the reabsorption of sodium and chloride at physiological rates of hormone administration is also important even though this effect is not invariable. Excessive, unphysiological amounts of the hormone were used in previous demonstrations (1-3) and hence the results of these are of questionable significance. Since the antidiuretic hormone may depress, and the suprarenal cortical hormone may enhance, the renal tubular reabsorption of sodium, both hormones are involved in the control of sodium balance. However, the action of the two does not appear to be integrated in the ordinary sense of the word. Relief of physiological oliguria by moderate amounts of water is usually accompanied by an increase in glomerular filtration rate (11) which involves an equivalent increase in the filtration of sodium. A deficiency of the antidiuretic hormone during such diuresis may be expected to enhance the reabsorptive capacity of the proximal tubule for sodium and thus permit water to be discarded and sodium to be retained (*cf* electrolyte excretion Table II). In this view, the hormone is important in the regulation of water balance *per se* and only incidentally of sodium. It is difficult to see how the acute changes which are involved in such responses can be closely integrated with the more stable influence of the suprarenal cortical hormone in the control of sodium reabsorption and excretion.

These experiments again emphasize the balance that normally obtains between glomerular and proximal tubular function and the consequences of a variation in one or another in the ultimate determination of the quality and quantity of urine formed (4, 5, 8). A direct demonstration of the relationships has not been achieved nor does it seem possible until more is known about the renal tubular reabsorption of sodium. However, the experimental data presented here and in the accompanying paper (8) lend strong support to the concept. Similarly, the loci of action of the antidiuretic hormone cannot be simply defined. However, to place its sodium effect in the proximal segment and its direct water reabsorptive action, *i e* the active process, in the distal, is in keeping with other evidence (6) and with our own experimental data.

SUMMARY

1 The administration of the posterior pituitary antidiuretic hormone by constant intravenous infusion has been used to examine the two characteristic actions of the hormone, namely, the facilitation of the active renal tubular reabsorption of water distally in the nephron and the inhibition of the renal tubular reabsorption of sodium proximally.

2 Experimental evidence was obtained which indicates that variations in the excretion of water and electrolyte involve the integration of these two actions with obscure variables which are discernible in the experimental data but are not subject to definition at this time.

3 Graded antidiuresis in the animal with diabetes insipidus, when normally hydrated, was only obtained in the range of 0.001 to 0.005 unit (pressor) per hour. This range of hormone administration was also found to be physiologically active in the normal animals. These observations together with others permit the placing of the normal rate of liberation of the antidiuretic hormone in a 10 to 15 kilo dog in the range of 0.001 to 0.005 unit per hour.

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QUANTITATIVE ALTERATIONS IN THE HYPEREMIA RESPONSES TO LOCAL ISCHEMIA OF THE SMALLEST BLOOD VESSELS OF THE HUMAN SKIN FOLLOWING SYSTEMIC ANOXEMIA, HYPERCAPNIA, ACIDOSIS, AND ALKALOSIS*

By JOSEPH R. DiPALMA M.D.

(From the Department of Physiology and Pharmacology, Long Island College of Medicine, Brooklyn)

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Little is known of the nature of the relationship of the concentration of blood gases, and of changes in pH of the blood to the responsiveness or state of tonus of the finest blood vessels (*i.e.*, the vessels beyond the arterioles). Investigations of a qualitative nature have led to the general belief that acids and carbon dioxide cause dilatation of the capillaries while alkalis and oxygen cause constriction (1-3). However, such experiments were largely confined to the local application of these substances in unphysiological concentrations. They suffered, moreover, from the added disadvantage that they were performed, for the greater part, upon lower animals, such as the frog. The development of a simple method for measuring quantitatively the hyperemia responses of the smallest blood vessels of the intact skin to local ischemia has provided the means for studying the reactivity of these vessels following systemic alterations of blood gases and changes in blood pH in human beings (4).

Methods and Procedures

Quantification of Reactive Hyperemia—The apparatus and methods of using this test have been fully described. Individual physiological variables seasonal and segmental differences have likewise been described recently (4, 5). Therefore, a brief account for purposes of orientation will now suffice.

Upon the skin of the ventral surface of the forearm, three rubber rings with surface areas of 5 sq. cm. each and with a weight loading of 100 gm. per sq. cm., are simultaneously applied. This is achieved with the aid of a suitable arm rest to which the weights are attached by a lever system. With this they can be lowered gently and accurately upon the skin. It will be seen that this procedure causes an ischemia of the dermal blood vessels immediately beneath and only beneath, the rubber rings. After a period of time measured in seconds the weighted rings are removed and the resulting areas of reactive hyperemia are carefully observed. If the period of application has been just sufficient to produce a ring of hyperemia localized exactly to the area of application of the rings and if these, in turn, have discrete edges and are of even intensity and color the *threshold* expressed in seconds is obtained. The length

* This investigation has been aided by grants from the Josiah Macy Jr., Foundation and the Committee on Endocrinology National Research Council

of time in seconds the threshold response requires to fade to the color of the surrounding skin is known as the *clearing* time. When the period of ischemia is less than threshold, an area of hyperemia results whose edges are diffuse and which is mottled, when the period of ischemia is greater than threshold, the hyperemic response is very intense, extending beyond the exact area of application, and a flare is present. With quickly acquired experience, the observer soon learns to read the threshold responses with a ± 3 per cent error. The use of three separate weights allows comparison of three results simultaneously, thus contributing to the accuracy of the reading.

A seasonal variation has been found to exist in this response (4). Thus, while the threshold is 10 to 15 seconds in midsummer, it may rise to 70 or 80 seconds in midwinter with corresponding greater rises in the clearing times. Since in this investigation the data were collected within a period of 2 weeks (late fall), the seasonal variable does not enter as a significant component.

All observations were made in a room with a temperature of about 75°F. Reading of the hyperemia responses was facilitated greatly by use of a blue mazda light bulb. The subjects were seated comfortably and were completely rested before each determination.

The advantages of using a method such as this for quantitating the responses of the small dermal blood vessels are several. It is easy and quick to perform, thus making these experiments possible. The threshold is an indication of the responsiveness (or state of tonus) of the smallest dermal blood vessels for it measures the amount of hypothetical "H" substance and collection of metabolites consequent to ischemia necessary to cause a certain amount of reactive hyperemia (6). In addition, the clearing time affords a direct indication of the rate of blood flow in these small dermal vessels (4, 5).

Experiments on Anoxemia and Hypercapnia—The subject breathed room air from a closed 72 liter tank. Carbon dioxide was absorbed from the expired air by a soda-lime cartridge. A tambour on one end of the tank permitted mensuration of respiratory rate and excursion. The volume of oxygen removed was measured by adding water to the tank at intervals exactly replacing the amount the subject utilized. From this the volume per cent of oxygen in the inspired air could be calculated. Blood pressure and pulse rates were taken at 5 minute intervals along with each determination of the hyperemia response.

The experiments on hypercapnia were made in exactly the same way as the anoxemia experiments except that the carbon dioxide of the expired air was not removed. Estimations of the carbon dioxide tension of the air in the tank were made at 5 minute intervals by the method of Marriott.

Experiments on Acidosis and Alkalosis—All subjects went without breakfast or any meal throughout the experiment. They were allowed to drink as much water as desired. The bladder was emptied early in the morning. 1½ hours later the bladder was emptied again. This was the control urine. During this interval the control observations on reactive hyperemia were also made. Now, the subject took either 5 to 10 gm of ammonium chloride or 10 to 15 gm of sodium bicarbonate to produce either acidosis or alkalosis. Observations on reactive hyperemia were made at ½ hour intervals for 5 hours. The subject also emptied his bladder at each ½ hour interval.

To test the disturbance in acid base equilibrium in each subject it was found most practical to analyze the urine specimens for total acidity and urinary ammonia and amino acids. This was done by the usual method of titration with 0.1 N sodium hydroxide to an end point using phenolphthalein as indicator then addition of neutralized formalin and titration to the second end point. The results were computed for the total volume collected in each interval.

RESULTS

Anoxemia—Nine experiments were performed on subjects (seven males and two females), all in the third decade of life except one who was in the fourth decade. The data obtained were averaged and compiled into one composite chart (Fig. 1). Since all the results in these experiments were in the same direction, such a chart of the mean results of nine individual experiments may be taken as a reliable indication of the average expectancy of such experiments.

Both the threshold and clearing time began to rise immediately and before any changes in heart rate, blood pressure, or respiratory rate and depth were noted. This rise continued steadily with the falling oxygen percentage of inspired air. At the termination of the experiments, when an average concentration of oxygen of 9 volumes per cent in the tank air was reached, corresponding to an altitude (with respect to per cent of oxygen, but not pressure) of approximately 22,000 feet, there had been a more than 30 per cent rise in the threshold and nearly a 50 per cent rise in the clearing time of the hyperemia responses.

Thus it is evident that systemic anoxemia causes a marked decrease in the sensitivity of the small dermal blood vessels to local ischemia. Since the clearing time has been shown to be directly related to the blood flow in these small vessels (4, 5), the increased clearing time obtained in this instance indicates a considerable decrease in the rate of blood flow in these fine vessels.

Hypercapnia.—Again the data from nine separate subjects in the third decade of life (eight males and one female) were compiled into a composite chart (Fig. 1). There is a slight rise in threshold and a greater rise in clearing time at the end of the first 5 minutes. Thereafter there is no further rise in threshold and an actual decrease in the clearing time which continues until the experiment is terminated. At this time the concentration of carbon dioxide in the inspired air has risen to 45 volumes per cent.

It is notable that despite the remarkable rise in pulse rate, blood pressure, and respiratory rate and depth, the responses of the smallest blood vessels remained practically unchanged throughout the experiment after the initial changes. This, along with the fact that changes in the responses of the small blood vessels occurred in the anoxemia experiment before any changes in pulse, blood pressure, and respiratory rate had taken place, demonstrates the independence of action of the small dermal blood vessels as contrasted with the functions of the arterioles.

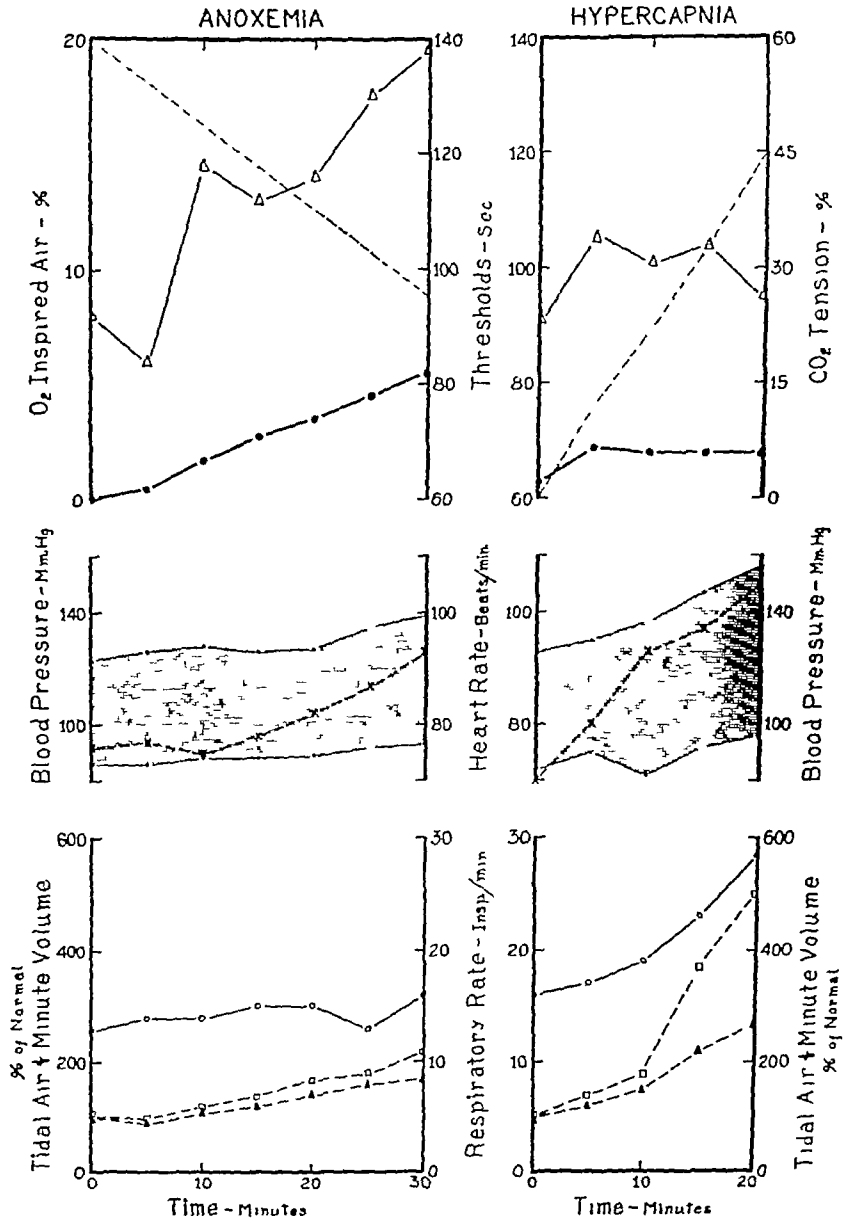


FIG 1 Top, the effects of anoxemia and hypercapnia upon the threshold (solid dots) and clearing time (blank triangles) expressed in seconds of reactive hyperemia in the skin. Middle, stippled areas indicate pulse pressure, crosses and discontinuous line, the heart rate. Bottom, continuous line, respiratory rate, squares, minute volume index (per cent), triangles, tidal air (per cent). (See text for discussion.)

It can be concluded, therefore, that retention of carbon dioxide in the blood completely neutralizes the effects of anoxemia upon small blood vessel responsiveness. Indeed, it renders them more sensitive in other ways, for this was proven in a previous experiment upon the effects of hypercapnia on the responsiveness of these small vessels to graded mechanical stimulation (7)

Acidosis and Alkalosis—In order to further elucidate the mechanisms by which variations of the concentration of blood gases cause changes in small dermal blood vessel responsiveness, experiments were performed on systemic acidosis and alkalosis as described above. Again it was decided to make a composite chart to simplify visualization of the data. Eight individual subjects all in the third decade of life, were used in each experiment. Fig 2 shows the thresholds and clearing times of the hyperemia test plotted at 30 minute intervals for the period of the experiment. The combined units (equivalents, in cubic centimeters, of 0.1 N acid) of urinary total acidity plus urinary ammonia also plotted indicate the direction and magnitude of the disturbance in acid base balance.

In the experiment on alkalosis (ingestion of 10 to 15 gm. of NaHCO_3), it is seen that there is an immediate fall in the threshold and an even greater fall in the clearing time at the end of 30 minutes. The fall in the threshold continues to the lowest point at the end of 120 minutes then it rises slightly, reaching a constant level at 210 minutes and continues at that level until the end of the experiment. The thresholds and clearing times follow rather well the changes in the total excretion of urinary total acidity and ammonia. The slight discrepancies may be explained by the lag which exists when the urine is analyzed at 30 minute periods. When the experiment was terminated the excretion of acid bodies in the urine was below the control level as was the threshold and clearing time. Thus it may be concluded that systemic alkalosis produced by ingestion of sodium bicarbonate causes an increased sensitivity of the smallest cutaneous blood vessels to local ischemia and an increase in the rate of blood flow through the skin as indicated by the shorter clearing times.

A similar experiment was performed again except that 5 to 10 gm. of ammonium chloride were administered to the subjects, thus producing systemic acidosis. Fig 2 shows that, again, the thresholds and clearing times of the hyperemia responses parallel the total excretion of acid bodies in the urine. Again the changes in the responses reached a maximum at 120 minutes after ingestion of the salt. At this time, the excretion of urinary total acidity and ammonia has also reached a maximum. At 210 minutes the hyperemia responses had again reached control levels although the excretion of acid bodies in the urine continued to be higher than the control.

Thus, it may be concluded that systemic acidosis, produced by the ingestion of 5 to 10 gm. of ammonium chloride, causes a decrease in the sensitivity of the smallest cutaneous blood vessels to respond by reactive hyperemia to local

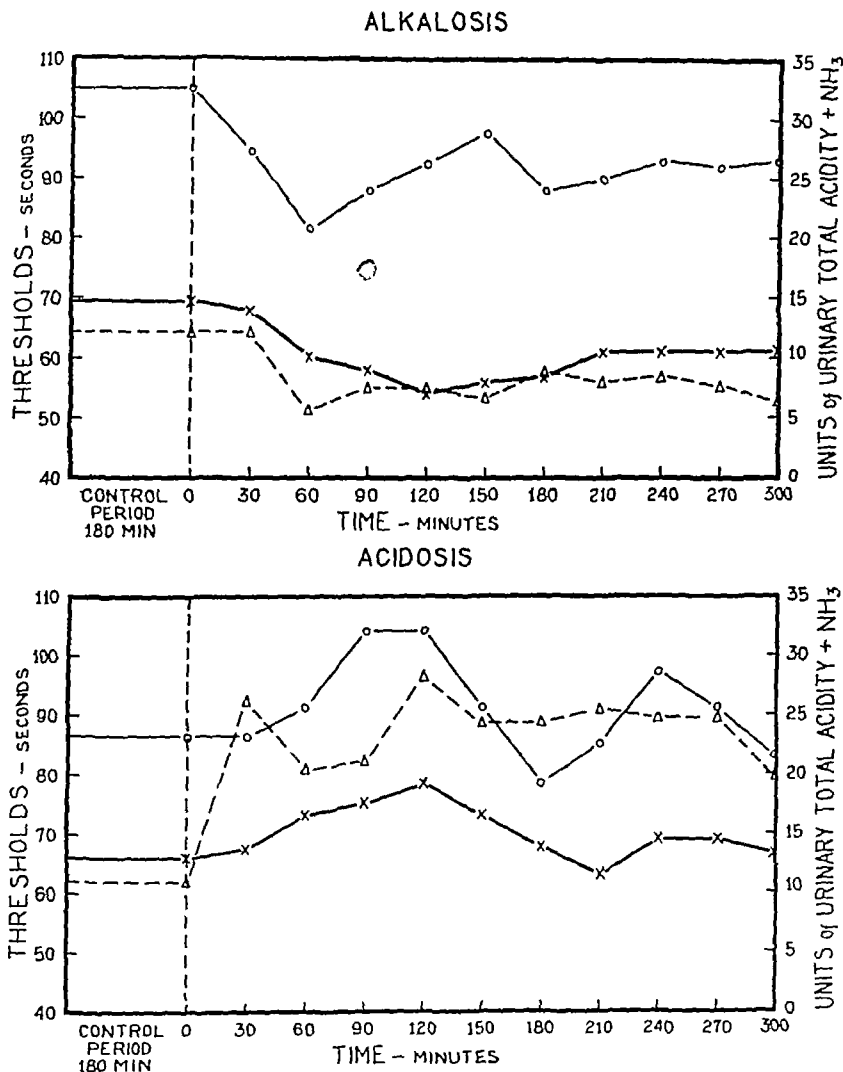


FIG 2 The effects of alkalosis (ingestion of NaHCO_3) and acidosis (ingestion of NH_4Cl) upon the threshold (crosses) and clearing time (circles) expressed in seconds of reactive hyperemia in the skin. Units of urinary excretion of total acidity plus urinary ammonia expressed as cubic centimeters of 0.1 N NaOH are also plotted (triangles). Note that maximum effects are obtained in both experiments at the 120 minute interval. (See text for discussion.)

ischemia, with corresponding decreases in blood flow as demonstrated by the higher clearing times.

The results of these two experiments are summarized in Table I to show the

carbon dioxide content of the blood also occurred (11). The definite changes in the excretion of urinary total acidity which we obtained in our experiments can, therefore, be taken to mean actual changes in acid base balance of the blood.

The only apparent explanation which will permit correlation of the anoxemia and hypercapnia with the acidosis and alkalosis experiments is that the concentration of carbon dioxide, held presumably as H_2CO_3 and NaHCO_3 , is the most important single factor in the responsiveness of the small cutaneous blood vessels as we have tested it. Thus it is seen that in the anoxemia experiment in which the carbon dioxide was eliminated and removed by soda lime, the sensitivity of the small dermal blood vessels decreased. In the hypercapnia experiment carbon dioxide was retained and, even though a considerable degree of anoxemia was also present, the small dermal blood vessel sensitivity remained unchanged. With alkalosis resulting from bicarbonate ingestion, carbon dioxide was retained to neutralize the increase in fixed bases in the blood, and the small dermal blood vessel sensitivity accordingly increased. Acidosis was accompanied by an elimination of carbon dioxide with a consequent decrease in small cutaneous blood vessel sensitivity. Similar results were obtained with respect to the contractile irritability of the cutaneous blood vessel reactions in response to graded mechanical stimulation in anoxemia and hypercapnia (7). The irritability and blood flow of the smallest vessels of the skin are directly related, therefore, to the concentration of carbon dioxide in the blood. The evidence Gesell (12) has recently advanced which demonstrates that increases in tissue acidity produced by increases in blood carbon dioxide cause the actions of acetylcholine to be prolonged, lends added credence to this hypothesis.

The mechanism by which the concentration of carbon dioxide controls the above mentioned phenomenon cannot be explained at this time but this does not detract from the practical application and implications of the results. For example, McConnell and Weaver (13) observed that ingestion of ammonium chloride in dogs diminished the size of histamine wheals while ingestion of sodium bicarbonate increased their size. They attempted to explain the results by a disturbance in calcium metabolism. In the light of the present experiments, it is possible that the changes in the histamine wheals which they observed resulted from the alterations in the small dermal blood vessel reactivity and blood flow consequent to the changes in blood pH.

Rous and Drury (14) have produced evidence that a condition of local acidosis exists in the skin of an extremity whose blood supply has been greatly reduced. In advanced arteriosclerosis obliterans of the extremities marked decrease in blood flow may occur. It was recently demonstrated, however, that in this disease the functional capacity of the finest skin vessels to respond by reactive hyperemia is not impaired. Evidently collection of other metabo-

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By PAUL GYÖRGY M.D. RUDOLPH TOMARELLI,*
ROBERT P. OSTERGARD M.D. AND
J. B. BROWN Ph.D.

(From the Babies and Childrens Hospital, and the Department of Pediatrics School of Medicine, Western Reserve University, Cleveland and the Department of Physiological Chemistry Ohio State University Columbus)

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Hepatic injury and the occurrence of malignant hepatoma after ingestion of butter yellow were first seen in rats (1) fed a diet of which 95 per cent was rice and 5 per cent olive oil. To this mixture the butter yellow was added in the proportion of 0.06 gm. per 100 gm. This ration was supplemented daily with a small amount of carrots. In contrast, practically no malignant changes have been seen (2), at least up to 150 days of the experimental period, in the livers of rats fed a diet (called diet C) that contained butter yellow in the same proportion as the original rice diet (0.06 per cent) but that had a different composition, i.e., casein 6 per cent, lard 23, cane sugar 15, cornstarch 50, salt mixture 4, and cod liver oil 2 and was supplemented daily with thiamine, riboflavin, pyridoxine, and pantothenic acid. The lesions in the livers of rats fed this diet were limited to necrosis, cirrhosis, and proliferation of the bile ducts, mostly typical in character, indeed only rarely atypical. Even these pathological changes were prevented to a large extent by the administration of a combination of cystine and choline (2).

Explanation of the difference in the effect of the rice-oil mixture and diet C on the production of malignant hepatoma in rats has been sought by feeding rats a modification of diet C. Crisco or melted butter fat was substituted for lard in one group of experiments and rice (brown unpolished or white) for cornstarch and sugar in another group of experiments. The effects of these substitutions proved in both sets of experiments to be procarcinogenic, especially in those in which brown rice was used. Furthermore, the malignant changes that followed ingestion of the rations containing butter fat or brown rice were not prevented, or were prevented only to a limited degree, by the administration of a combination of cystine and choline.¹

The problem that arose, therefore, was to learn what principle was operating

* S.M.A. Corporation Fellow

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lites, including carbon dioxide and possibly the hypothetical "H" substance, prevents the effects of local acidosis from playing a dominant rôle in such a condition

It is also interesting to speculate on the effects of prolonged periods of systemic acidosis such as occur in uncontrolled diabetes mellitus, upon the smaller skin circulation. In the light of the present findings of diminished reactivity of the small dermal blood vessels in systemic acidosis, one wonders if the acidosis of diabetes contributes, in this way, to the susceptibility of these patients to skin infections and degenerative dermal changes

SUMMARY AND CONCLUSIONS

The responsiveness of the smallest blood vessels of the human skin was measured in systemic anoxemia, hypercapnia, acidosis, and alkalosis. A method was used which measured quantitatively the reactive hyperemia produced by a standardized period of local ischemia of these fine vessels. By timing the clearing period of the threshold hyperemia response a direct indication of blood flow in these fine vessels was obtained. The following conclusions were reached concerning the responses of the smallest blood vessels of the skin:

- 1 Systemic anoxemia causes a decrease in sensitivity to local ischemia and a slowing of the blood flow.
- 2 Hypercapnia prevents the changes resulting from anoxemia.
- 3 These changes in the smallest blood vessels of the skin occur independently of changes in pulse rate, blood pressure, and respiratory rate and depth.
- 4 With systemic acidosis there is a decrease in sensitivity to local ischemia and a slowing of blood flow. The exact opposite takes place in systemic alkalosis.
- 5 The view is advanced, after due consideration of the facts, that the carbon dioxide concentration of the blood, or something directly associated with it, is the most important factor determining the sensitivity of these vessels, rather than oxygen saturation or changes in blood pH.

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The problem that arose, therefore, was to learn what principle was operating

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in the change of the non-carcinogenic diet C into a carcinogenic ration following the incorporation in it of crisco or butter fat in place of lard in one set of experiments and of rice (white and especially brown) instead of cornstarch and sugar in another. The answer has been provided by results of still other experiments in which the source of fat was, first, a crude preparation of linoleic acid² and, later, pure specimens of fatty acids and their esters, prepared in the laboratory of one of us.

EXPERIMENTAL

The experimental results, representative examples of which are given in Tables I and II, can be summarized as follows —

1 Diet C with crude linoleic acid replacing lard (referred to as diet "By" in Tables I and II) proved to be toxic for rats weighing over 130 gm at the beginning of the experiment. Sixty-seven rats were fed this diet in amounts *ad libitum*, and the results were consistent. The animals lost weight rapidly in spite of satisfactory intake of food. They exhibited marked, progressive anemia, secondary in type, which was accompanied, as a rule, by leucopenia (Table I). They became infested with pediculi, often before the development of anemia when the animals were still very active and not nearly moribund.

The toxic effect on rats of the ration containing linoleic acid was even more pronounced when butter yellow was omitted from diet "By" (referred to as diet "L₁" in Table I). In this group comprising 23 rats all the animals died before the 80th experimental day, with an average survival time of 55 days. Four rats showed leucopenia but no anemia before death.

The toxic effect of the rations containing linoleic acid with ("By") or without ("L₁") butter yellow could, as a rule, be neutralized preventively and therapeutically by the daily addition of 0.5 to 1 gm of yeast. Twenty-one rats were fed diet "By" and 11 rats were fed diet "L₁". The observation period for these groups was extended to 150 days. In 3 of the 11 rats fed ration "L₁" (without butter yellow) the anemia was not prevented by addition of 1 gm of yeast daily, and the animals died before the end of the experimental period.

In tests involving 49 rats, a diet containing brown rice, linoleic acid, and butter yellow was found to be non-toxic. On the other hand, linoleic acid was almost completely oxidized and destroyed by keeping diet "By," before use, for 3 to 4 weeks at laboratory temperature, and the diet then exerted marked toxic effect on all 23 rats in this group. Diarrhea was a prominent feature, in addition to loss in weight, anemia, leucopenia, and pediculosis.

Postmortem examination revealed that rats fed the modification of diet C with linoleic acid instead of lard and with or without butter yellow were generally free from pathological changes in the liver, even from severe fat infiltration. Animals receiving diet C with linoleic acid and butter yellow never showed malignant hepatoma. The life span of these rats could be prolonged by daily

² "Linoleic acid refined light" from the Gluco Products Co., Inc., Brooklyn, N. Y.

TABLE I

Morphological Blood Changes in Rats Fed Rations Containing Crude Linoleic Acid

Rat No and date when experiment was started	Diet	Blood examination								
		Date	Hb	Red blood cells $\times 10^6$	White blood cells (corrected) $\times 10^6$	Reti- cytes	Nucleated red cells	Polymorpho- cytes	Small lymphocytes	Large lymphocytes
			per cent			per cent	per cent	per cent	per cent	per cent
7596—12/2	‘By’ + cystine + choline	2/18	72	5.7	9.4	0.3	1	20	76	4
		3/13	46	2.25	7.8	22.5	6	22	78	
		4/3	36	2.6	4.5	5.6	20	12	88	
		4/28	15	1.05	5.4	17.0	32	14	84	2
8063— 3/3	‘By’	3/25	93	6.1	26.9	3.2		16	80	4
		4/17	70	6.35	27.0	0.9	6	12	88	
		5/8	59	4.45	9.3	0.4	20	24	76	
		5/19	16	1.1	6.8	—†	40	20	80	
8132— 3/19	‘By’ + yeast	4/7	83	7.2	19.2	9.6	2	20	80	
		4/30	88	7.35	12.5	2.6	2	36	64	
		5/21	84	5.5	18.5	4.4	4	28	68	4
6995—11/17	‘By’ + cystine and beginning 1/29 + yeast	1/29	29	3.0	2.3	—†	2	34	58	8
		2/16	63	6.4	3.4	2.1	52	48	52	
		3/31	78	5.7	14.8	7.6		28	72	
		5/11	79	6.65	11.2	7.2		34	66	
8007— 2/21	‘Li’	2/23	94	8.1	15.2	0.3		8	88	4
		3/17	90	6.6	18.2	0.9		10	88	
		4/21	45	4.55	4.5	1.2	2	20	76	4
		4/28	13	1.1	0.24	16.0	85	85	15	2
8011— 2/21	‘Li’ + yeast	3/20	92	8.0	12.8	9.6		24	76	
		4/13	107	8.25	7.8	2.4		20	80	
		5/5	103	8.4	19.0	0.2		14	86	

* Diet ‘By’ casein 6 per cent, cornstarch 50, sucrose 22, cod liver oil 2, salt mixture 4, linoleic acid 16, and butter yellow 0.06. Diet ‘Li’ same as ‘By’ without butter yellow. Both diet ‘By’ and diet ‘Li’ were supplemented daily with 20 μ g. of thiamine, 25 μ g. of riboflavin, 20 μ g. of pyridoxine, and 100 μ g. of pantothenic acid. Both rations were freshly prepared twice a week.

† Not examined

supplements of cystine (50 mg) or of cystine (50 mg) plus choline (20 mg). Only one of the 19 rats that did not receive supplements of cystine or choline survived for 100 days. The average survival time in this group was 61 days.

why at the same time a lard diet does not entail toxic manifestations, such as are seen after administration of a diet containing linoleic acid, needs further study for its elucidation

If these considerations are correct, the procarcinogenic effect of crisco or butter fat as well as that of rice could be explained by the preservation of butter yellow in the diet and the intestine because of a low intake of unsaturated fatty acids, in the case of butter fat or crisco, and by the presence of a stabilizer or antioxidant, in the case of rice

According to the literature (4, 5) the production of butter yellow cancer is hindered by administration of rice bran oil obtained by extraction from rice bran with ether. This fact puts rice bran oil in the same category as lard. Furthermore it tends to corroborate the assumption that the antioxidant of rice is not fat-soluble and does not pass into the ether extract

The metabolism of *N,N*-dimethylaminoazobenzene has hitherto been regarded as a cellular process (6). The investigations reported here throw light on a reaction which occurs without the participation of living tissue. In the light of these findings it is noteworthy that recently the destruction of hemoglobin and hemin (7) and of carotene (8) by linoleic acid has also been reported. It remains to be shown whether the conservation and destruction of butter yellow in the diet and in the intestine, as illustrated by the investigations here analyzed, exhaust all possibilities of the influence of diet on "butter yellow cancer." No such claim is presented here.

Recently great interest has been aroused by the identification of biotin as a procarcinogenic agent for butter yellow cancer (9). In the relevant experiments of du Vigneaud and his collaborators biotin was given by mouth and thus it may have acted locally in the dietary mixture or in the intestine as stabilizer for butter yellow, an effect similar to that of rice. In preliminary experiments, however, which are being continued, we have been unable to demonstrate such an effect *in vitro*.

The production of progressive anemia and leucopenia when linoleic acid is contained in the diet and their prevention by the administration of yeast recall the old hypothesis concerning the rôle ascribed to unsaturated fatty acids, mainly oleic acid, in the pathogenesis of pernicious anemia and anemia caused by tapeworm (*Bothriocephalus latus*) (10). The fact that in these previous experiments anemia could be produced by parenteral, but not by oral, administration of oleic acid has never been explained. That essential fatty acids or rather, probably, some of their oxidative break-down products, exert an injurious effect on hematopoiesis only if the diet is deficient is a fact not known hitherto and one which has become evident only in the present experiments. Further studies are needed in order to identify this deficiency which, together with the pathogenesis of "butter yellow cancer," is a good illustration of the

often recurring problem whether intoxication or deficiency (11) plays the leading part in a given dietary disturbance

The special type of anemia and the cancer of the liver found in the present investigations were mutually exclusive. In this connection it is illuminating that in certain races of the Far East primary cancer of the liver is a common disease whereas pernicious anemia occurs very rarely (12). It should be emphasized, however, that the morphologic blood picture of pernicious anemia differs from that seen in rats fed a diet containing linoleic acid, the latter anemia being of the secondary type. This difference may be due either to the differing response of man and rat or to the differing pathogenesis.

SUMMARY

Crude linoleic acid incorporated with or without butter yellow in a synthetic diet proved to be toxic for rats. The toxic effect manifested itself in loss of weight, progressive anemia of the secondary type, leucopenia, and pediculosis. It could be neutralized preventively and therapeutically by administration of yeast. The toxicity of the diet containing linoleic acid appears to be due to oxidative break-down products of the unsaturated fatty acid.

The color of the same diet when it contained crude linoleic acid supplemented with butter yellow faded progressively in the presence of air (O_2), even at room temperature. Purified preparations of linoleic acid and, to a less degree, purified preparations of arachidonic and oleic acids have shown the same destructive effect on butter yellow *in vitro*.

Brown (unpolished) or white rice contains a stabilizer (antioxidant) for the preservation of butter yellow.

In experiments on the production of hepatoma in rats following the ingestion of butter yellow, rice on one hand and crisco or butter fat on the other hand have proved to be procarcinogenic. These results would seem to be correlated with the preservation of butter yellow in the diet and in the intestine, because of the antioxidant in rice and the low supply of unsaturated fatty acids, respectively.

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LIVER INJURY, LIVER PROTECTION, AND SULFUR METABOLISM

METHIONINE PROTECTS AGAINST CHLOROFORM LIVER INJURY EVEN WHEN GIVEN AFTER ANESTHESIA*

BY L. L. MILLER, PH.D. AND G. H. WHIPPLE, M.D.

(From the Department of Pathology of The University of Rochester School of Medicine
and Dentistry, Rochester New York)

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The experiments listed below may seem unrelated, but, in fact, bear on the question of the defense mechanism of the liver cells against chloroform injury. It came as a complete surprise to us when experiments showed that methionine gave considerable protection against liver injury even when given 3 to 4 hours after the anesthesia.

The observed fact that the livers of fetuses or of newborn pups showed unexpected tolerance to chloroform injury has long interested us. The newborn pups exhibit remarkable tolerance to chloroform injury and this peculiar quality lasts 2 to 3 weeks after birth, as shown years ago by one of us (20). Tables 3 and 2 show that the nitrogen sulfur ratio of pups and normal adults is about the same, that is, 11 or 12 to 1. The protein-depleted dog which is so susceptible to liver injury, shows a nitrogen sulfur ratio of 14 or 15 to 1, which means presumably less available sulfur-containing amino acids.

It is obvious, too, that during long protein depletion (4 to 8 weeks) on a very low protein diet, the liver is drained of its sulfur a little more completely than of its nitrogen. This sulfur deficit in the liver can be made up very promptly by feeding methionine and less effectively by cystine.

Previous reports (13, 14) from this laboratory have shown that the protein depleted dog is extremely susceptible to chloroform liver injury, and that a single large protein feeding protects the protein-depleted dog from an otherwise fatal chloroform anesthesia and related liver injury. It was then shown (14) that *dl* methionine and, to a less extent, *l*-cystine, given before chloroform anesthesia, are as effective in protecting the protein-depleted dog as a large protein feeding. A variety of non-sulfur-containing amino acids were found to be entirely lacking in this protective action.

Methods

Chloroform Anesthesia Experiments—All dogs used in these experiments were active healthy adults. As indicated in detail in the individual protocols the dogs' reserve stores of protein were depleted by maintenance on a very low protein diet con

* We are indebted to Eli Lilly and Company for aid in conducting this work.

sisting of sucrose 72.2 per cent, salt mixture (19) 4.6 per cent, bone ash (largely calcium phosphate) 4.6 per cent, crisco 14.9 per cent, mazola oil 6.5 per cent, cod liver oil 1.4 per cent, yeast powder (Fleischmann's Type 200-B), 0.7 per cent, powdered liver extract (Lilly, H8083), 0.7 per cent, nicotinic acid 13.9 mg per cent, choline chloride 111 mg per cent. The amount of protein in this diet did not exceed 1½ gm per 100 gm as fed.

The amounts of *dl*-methionine (Merck and Co),¹ *l*-cystine (Eastman Kodak), and choline chloride (Eastman Kodak), and the routes of administration are given in the individual protocols. Other methods are described in previous reports (13, 14).

Analysis of Livers—In all cases the livers were rapidly removed, and the excess blood or perfusion fluid removed by gently blotting with filter paper. The livers were then cut into small pieces, weighed, and dried to constant weight at 76°C (usually about 72 hours). The dried liver was then ground to a powder and samples taken for analysis.

The total nitrogen determinations on liver tissue were done by macro-Kjeldahl, and the total sulfur by the wet ashing of Masters (11), using concentrated nitric and perchloric acids, followed by precipitation of sulfate as barium sulfate.

EXPERIMENTAL OBSERVATIONS

In experiments previously reported it was shown that as little as 3.0 gm of *dl*-methionine given *before* anesthesia would protect completely the protein-depleted dog from the effects of a 40 minute chloroform anesthesia. This was in distinct contrast to the almost invariably fatal liver damage following only a 20 minute anesthesia in the unprotected protein-depleted dog.

Experiments 1 and 2 show a totally unexpected response. Methionine given intravenously 3 and 4 hours after the start of a 30 minute chloroform anesthesia prevents the fatal injury, but does not prevent some liver damage. On the basis of clinical condition, icterus index, and blood fibrinogen levels, dog 40-251 which received methionine 4 hours after chloroform anesthesia presented the picture of the more severe liver injury.

In Experiment 3, dog 40-251 was moderately protected by cysteine plus choline given intravenously 3 hours after chloroform anesthesia. It is interesting to note that the blood fibrinogen level did not fall below 250 mg per cent despite the apparently severe liver damage. This is probably related to the presence of several purulent ulcers of the skin, as it is well known that suppuration raises the level of blood fibrinogen, even in the presence of chloroform liver injury.

¹ We are indebted to Merck and Co for valuable amino acids.

TABLE 1

*Methionine and Cysteine Given 3 to 4 Hours after Chloroform Protects Liver**Experiment 1 (dog 39-230) Methionine gives moderate protection*

Time before and after chloroform	Fibrinogen	Icterus index	Clinical condition
hrs	mg per cent		
0	420	0	Normal (plasma protein = 5.10 gm per cent)

30 min chloroform anesthesia, *dl* methionine given by vein 3 and 6 hrs. after start of anesthesia (total 3 gm.)

6	390	0	Normal
24	280	2	Good vomited some mucus
48	130	9	Excellent
72	140	13	'
96	187	11	'
120	240	6	recovery

Experiment 2 (dog 40-251) Methionine gives moderate protection

0	315	0	Normal (plasma protein = 5.06 gm per cent)
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30 min chloroform anesthesia, *dl*-methionine given by vein 4 hrs. after start of anesthesia (total 3.0 gm.)

24	210	8	Somewhat quiet but good
48	114	11	Good
72	102	17	
96	—	14	
216	282	3	Excellent recovery

Experiment 3 (dog 40-251) Cysteine + choline gives moderate protection*

0	260	0	Normal (plasma protein = 5.22 gm. per cent)
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30 min chloroform anesthesia, cysteine (2.85 gm. of hydrochloride) + choline hydrochloride (1.0 gm) given by vein 3 hrs. after anesthesia

24	250	13	Plasma protein 5.06 gm per cent Intoxicated vomited mucus, weak
48	250	16	Very weak vomited mucus. Skin ulcers
72	340	14	Improved. Ulcers purulent
96	410	14	Good recovery

* Interval of 18 wks. between Experiment 2 and Experiment 3

Clinical Histories

Experiment 1 dog 39 230 Mongrel hound Feb 22 1941—Weight 9.1 kg
Plasma protein level 6.32 gm per cent Placed on low protein diet, 135 gm. daily

Feb 23 to Apr 29—Ate an average of 60 to 80 per cent of food Apr 30—Fasted
May 1—Weight 8.5 kg Plasma protein level 5.10 gm per cent *Chloroform* anesthesia
—See Table 1 The *dl*-methionine in this and the following experiments was dissolved in about 150 cc of physiological saline and given intravenously May 1 to May 6—Ate an average of 10 per cent of diet May 7—Placed on kennel diet Weight 8.0 kg

Experiment 2, dog 40-251 Mongrel hound, male May 28 to June 9, 1941—Fasted Plasma protein level on June 5, 6.68 gm per cent, weight 12.2 kg June 10 to Aug 4—Low protein diet, 200 gm daily, eaten 95 to 100 per cent Plasma protein level, July 29, 4.85 gm per cent Aug 5—Fasted Aug 6—Weight 10.3 kg Plasma protein level 5.06 gm per cent *Chloroform* anesthesia—See Table 1 Methionine given 4 hours after chloroform Aug 6 to 15—Ate 95 to 100 per cent of diet except on Aug 7, when only 40 per cent was eaten

Experiment 3, dog 40-251 Nov 12 to Dec 13, 1941—On low protein diet, 200 gm daily, eaten 100 per cent Weight on Nov 14, 11.0 kg Dec 14—Fasted Noted a number of small ulcerated areas on skin pressure points Dec 15—Weight 9.7 kg Plasma protein level 5.22 gm per cent *Chloroform* anesthesia—See Table 1 (choline chloride and cysteine hydrochloride neutralized with sodium bicarbonate before being given intravenously, 0.6 mg atropine sulfate intramuscularly to control salivation) Dec 15 to Dec. 19—Ate practically no food Ulcerated areas frankly purulent Dec. 20—Returned to kennels

Experiments 4 and 5 show that *dl*-methionine, given intravenously as in the above experiments 4 and 6 hours respectively *after the start of the anesthesia*, fails to prevent fatal liver damage with typical severe hyaline central liver necrosis Evidently 4 hours is about the time limit beyond which methionine ceases to protect the liver against chloroform injury due to 30 minutes of anesthesia

Experiment 6 confirms previous observations (6), that relatively large amounts of choline given *before anesthesia* fail to prevent fatal liver damage

TABLE 1-a

Methionine Given 4 to 6 Hours after Chloroform and Choline Given before Chloroform Fail to Protect Liver

Experiment 4 (dog 40-40f) Methionine fails to protect when given 4 hrs after anesthesia

Time before and after chloroform	Fibrinogen	Icterus index	Clinical condition
hrs	mg per cent		
0	266	0	Normal (plasma protein = 4.99 gm. per cent)

30 min. chloroform anesthesia, methionine given by vein 4 hrs. after start of anesthesia (total 3.0 gm)

24 40†	100±*	20	Droopy Dead Liver extreme hyaline necrosis
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Experiment 5‡ (dog 30-230) Methionine fails to protect when given 6 hrs after anesthesia

0	387	0	Normal (plasma protein = 4.45 gm. per cent)
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30 min. chloroform anesthesia, methionine given by vein 6 and 20 hrs. after start of anesthesia (total 3.2 gm)

24 36‡	239	20	Fair Vomited mucus Dead Liver extreme hyaline necrosis
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Experiment 6 (dog 39-338) Choline before anesthesia fails to protect

0	355	0	Normal (plasma protein = 5.07 gm per cent)
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20 min. chloroform anesthesia, choline hydrochloride (2.0 gm) by mouth 3½ hrs. before anesthesia

24 34‡	209	13	Severely intoxicated vomited mucus Dead Liver, extreme hyaline necrosis
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* Poor clot in fibrin formation with calcium chloride.

† Estimated hour of death.

‡ Interval of 13 wks. between Experiment 1 and Experiment 5

Clinical Histories

Experiment 4 dog 40-40f Mongrel hound male. July 11, 1941—Weight 11.8 kg Started on low protein diet 200 gm daily following 10 days' fast. July 12 to July 31—Ate 90 to 100 per cent of food Aug 1—Ate 30 per cent of food Plasma protein level 5.03 gm per cent. Aug 2 to Aug 6—Added 5 gm fresh pig liver to daily diet Ate 90 to 100 per cent of food Aug 7—Fasted Aug 8—Weight 10.2 kg Plasma protein level 4.99 gm per cent. Chloroform anesthesia 30 minutes duration started 4 hours before methionine was given—See Table 1-a Aug 10—Found dead In the gross and microscopically the liver showed severe central necrosis

Experiment 5, dog 39-230 June 5—Returned to low protein diet following 10 days' fast Plasma protein level 5.45 gm per cent Weight 7.3 kg June 6 to July 28—Ate an average of 35 to 40 per cent of diet July 29 to Aug 4—Added 10 gm of fresh pig liver to daily diet Ate an average of 80 per cent of food Aug 4—Fasted Aug 5—Weight 5.8 kg Plasma protein level 4.45 gm per cent *Chloroform* anesthesia—See Table 1-a Aug 7—Found dead Autopsy reveals unclotted blood in great vessels and heart In the gross and microscopically the liver showed severe central necrosis

Experiment 6, (dog 39-338) Mongrel hound, male Oct 1 to Dec 20, 1941—Low protein diet 185 gm daily Ate average of 95 per cent of food Dec 20 to Dec 24—Ate 60 to 70 per cent of food Dec 24 to Jan 3, 1942—Added 10 gm fresh pig liver to daily diet Ate 95 per cent of food Jan 4—Ate 60 per cent of food Weight 11.1 kg Jan 5—Ate 50 per cent of food, choline chloride added to give 0.60 gm total ingested Jan 6—Ate about 50 per cent of food, choline chloride added to give 1.0 gm of choline chloride ingested Jan 7—Fasted Jan 8—Plasma protein level 5.07 gm per cent Gave 2.0 gm of choline chloride by stomach tube, preceded by 0.6 mg of atropine sulfate intramuscularly to prevent excess salivation *Chloroform* anesthesia 20 minutes started 3½ hours after choline was given See Table 1-a Jan 10—Found dead Autopsy reveals severe central necrosis of liver both in the gross and microscopically

Histological Specimens (Table 1-a)

Dog 39-404—Typical extreme hyaline central liver necrosis, a few liver cells about the portal structures are not necrotic Dogs 39-230 and 39-338 present an identical picture

In Table 2 are listed the nitrogen and sulfur analyses of *adult dog livers*. It is obvious that the liver N/S ratio in the *dog well fed with protein* is distinctly lower (10.5 to 12.4) than the liver N/S ratio in the *protein-depleted dog* (14.1 to 15.4). The feeding of *L*-cystine (dog 39-215) or *DL*-methionine (dog 39-164) lowered the *liver* N/S ratio markedly in both cases. The response to methionine feeding is greater though less total methionine was fed. The comparatively small change in the muscle N/S ratio makes it appear that the liver can hoard the S-containing amino acids in some form. It is not unlikely that much of the methionine is incorporated in the proteins of the liver cell.

In the adult dogs the milligrams of liver N per cent body weight in the protein-depleted dogs do not vary significantly from those of the normal well fed dogs. This may be attributed to the fact that, with long extended protein deprivation, the percentage decrease in total carcass protein probably reaches about the same value as the liver protein decrease. In rats, Addis, Poo, and Lew (2) have demonstrated essentially this fact.

TABLE 2
Liver Nitrogen and Sulfur
Normal Well Fed Adult Dogs

Dog No	Plasma protein level	Body weight	Liver weight	Liver solids	Dry Liver				
					N	S	$\frac{N}{S}$	Mg. N	Mg. S
	gm per cent	kg.	gm	per cent	per cent	per cent		per cent body weight	
5E		18.6	539	26.9	9.21	0.752	12.2	72	5.9
2F			306	24.8	10.64	0.885	12.0		
1F			320	23.4	10.58	0.860	12.3		
40-167		14.8	377	19.2	11.65	0.941	12.4	57	4.6
41 137	6.44	15.4	435	19.0	10.27	0.942	10.9	55	5.0
41 127	6.19	12.6	355	27.4	9.54	0.896	10.6	74	7.0
41 138	6.58	14.0	362	26.2	10.42	0.858	12.2	70	5.7
	(5.21)								
41 130	6.08	11.7	315	26.2	10.94	1.03	10.5	77	7.3
	(4.80)								
Average				24.1	10.41	0.896	11.6		

Protein Depleted Adult Dogs

39-10*	4.90	8.2	214	27.1	7.58	0.527	14.4	54	3.8
35-6	4.14	18.0	790	26.2	7.17	0.502	14.3	82	5.7
39-230	4.45	7.7	230	22.5	8.91	0.631	14.1	81	5.7
41-305			313	26.6	11.23	0.736	15.3	Pregnant	
41-308			377	33.0	7.02	0.454	15.4		
Average				27.1	8.38	0.570	14.7		

Protein Depleted Adult Dogs Fed Methionine or Cystine

39-164†	4.00	7.4	238	27.8	5.65	0.674	8.4	51	6.1
39-215‡	4.95	12.1	597	31.2	5.60	0.494	11.3	85	7.5

$$* \text{ Muscle } \frac{N}{S} = \frac{12.79}{0.72} = 17.8.$$

$$\dagger \text{ Muscle } \frac{N}{S} = \frac{11.40}{0.75} = 15.2 \text{ dl-methionine, 1.0, 1.0, 1.5 gm fed on 3 days respectively before sacrifice.}$$

$$\S \text{ Muscle } \frac{N}{S} = \frac{13.10}{0.83} = 15.8 \text{ L-cystine 1.0, 1.0, 1.0, 1.5 gm fed on 4 days respectively before sacrifice.}$$

Histological Specimens (Table 2)

Dog 41-137—Cells are normal, glycogen granules are obvious

Dog 41-127—Cells are normal, glycogen granules are conspicuous

Dogs 41-138 and 41-130—Cells are normal

Dog 39-10—Liver cells are normal Glycogen is conspicuous in liver cells in central half of lobule

Dog 35-6—Liver cells are normal, stuffed with glycogen and have the appearance of vegetable cells

Dog 39-230—Liver shows usual extreme liver necrosis

Dog 41-305—Fatal chloroform poisoning—usual extensive hyaline liver necrosis (pregnancy)

Dog 41-308—Fatal chloroform poisoning and liver injury (pregnancy)

Dog 39-164—Liver cells are stuffed with glycogen (like 35-6)

Dog 39-215—Liver cells are stuffed with glycogen (like 35-6)

Table 2 contains several individual experiments which deserve comment

The first 4 dogs (5E, 2F, 1F, and 40-167) had been used for radio iron metabolism experiments, were on a high protein intake, and were all young, active, and healthy adults They were then perfused under ether anesthesia Dogs 41-137 and 41-127 were normal dogs on a mixed diet, fasted 24 hours, and killed under ether after a perfusion which freed the liver tissue of blood These dogs show a normal plasma protein level and liver tissue which is normal histologically Although there is much difference in the amounts of liver solids, the N/S ratio is similar, and we suspect a "dilution" of the cell protein by fat or glycogen in dog 41-127

Dogs 41-138 and 41-130 were well fed dogs which had been subjected to a rapid and severe *plasma depletion* (bleeding and return of red cells suspended in saline) which had lowered the plasma protein levels from 6.58 to 4.23 gm per cent and from 6.08 to 3.58 gm per cent respectively After 24 hours the plasma protein levels had risen to 5.21 and 4.80 gm per cent respectively, presumably due to rapid influx of plasma protein from reserve body stores (liver and muscle) The dog was then perfused under ether anesthesia This procedure did not disturb the figures for nitrogen and sulfur in the liver

Protein-depleted dogs (Table 2) show many points of clinical interest

Dog 39-10 had been on a very low protein diet (not more than 2 to 3 gm protein per day) for 13 weeks and was exsanguinated under ether anesthesia The plasma protein level was low (4.90 gm per cent) and the percentage of N and S were both much below normal, but the S was the lower, giving a high N/S ratio, 14.4 Dog 35-6 was both anemic and plasma-depleted One week's protein fast was followed by 4 weeks of a very low protein diet (not more than 2 to 3 gm protein per day) The dog was then perfused under ether The plasma protein level was very low (4.14 gm per cent), and there had been a loss of 3.5 kilos body weight The N and S values are almost exactly those of the preceding experiment

In dog 39-230 (refer to Table 1-a and Clinical history), a 10 day fast was followed

by the low protein diet for 9 weeks. Plasma protein level (4.45 gm. per cent) was low and there had been a loss of weight of 2 kilos. Chloroform anesthesia 30 minutes caused death in $36 \pm$ hours. The liver showed typical extreme hyaline necrosis. The N and S analyses are much like those of plasma depletion alone. The susceptible and damaged liver cells evidently contain about the same amount of protein (Table 2). Methionine given 6 hours after chloroform was not taken up in significant amounts by these injured and necrotic liver cells—compare with dog 39-164 (Table 2) where methionine uptake is obvious.

Pregnant dogs 41-305 and 41-308 (Table 2) were obviously close to term. They were not well nourished and we know nothing about their dietary history. They were both fasted for 3 days. The dogs went into labor. Pups 308-1 and 2, and 305-1, 2, 3, and 4 were born (Table 3). The mothers were then given light surgical chloroform anesthesia—Dog 41-305 50 minutes and dog 41-308 70 minutes. Recovery from chloroform anesthesia was prompt and during the ensuing 4 hours pups 305-5c, 6c, and 7c and pups 308-3c, 4c, and 5c were born normally. The mother dogs died in about 15 hours following the chloroform anesthesia with ample evidence of fatal chloroform liver injury. The pups' livers exposed to the same dose of chloroform *in utero* showed not a trace of injury (Table 3 and Histological specimens).

Protein-depleted dogs 39-164 and 39-215 (Table 2) had been on a low protein diet for 9 weeks which had effected some hypoproteinemia (4.00 and 4.95 gm. per cent respectively). They were fed methionine and cystine (Table 2) and 24 hours later were exsanguinated under ether. The content of the dry liver in N and S is decidedly low as compared with normal dogs but the N/S ratio is decreased in comparison with protein-depleted dogs not fed the S-containing amino acids. Obviously the liver tissue has taken up these amino acids more than the muscle tissue and more of the methionine than of the cystine. The increase in liver solids is certainly in large part due to glycogen (Histological specimen). The low content of nitrogen *might* be due to metabolic activity related to the methionine and cystine intake—a turn over of protein to supply other body protein emergency needs. The S is largely retained in the body in such experiments (17).

TABLE 3
Liver Nitrogen and Sulfur
• Normal Pups (48 Hrs Old)

Pup No	Weight	Liver weight	Liver solids	Dry liver			
				N	S	$\frac{N}{S}$	$\frac{Mg}{N}$ $\frac{Mg}{S}$
				per cent body weight			
	kg	gm	per cent	per cent	per cent		
1	0 32	12 3	22 2	12 77	0 949	13 4	109 8 1
2	0 33	13 0	23 5	11 30	0 892	12 8	112 8 8
3	0 31	11 6	22 9	12 78	0 988	12 9	110 8 5
4	0 33	13 0	23 8	13 59	1 17	11 8	127 10 8
Average			23 1	12 61	0 998	12 7	

Pups Born before Chloroform Anesthesia (7 Hrs Old)

308-1	0 25	12 0	30 7	7 87	0 720	10 9	116 10 6
308-2	0 23	13 0	26 9	6 84	0 633	10 8	104 9 6
305-1 & 2	0 35	16 0	28 8	8 16	0 735	11 1	109 9 8
305 3 & 4	0 43	20 5	26 8	7 07	0 641	11 7	91 7 8
Average			28 3	7 48	0 682	11 1	

Pups Born after Chloroform Anesthesia (48 Hrs Old)

308-3 & 4c	0 42	15 6	24 5	12 34	1 07	11 5	113 9 8
308-5c	0 18	7 4	21 2	13 02	1 01	12 9	116 9 0
305-5c	0 14	10 0	22 1	12 40	1 03	12 0	203* 16 9
305-6 & 7c	0 39	17 9	22 5	11 37	0 967	11 8	118 10 0
Average			22 6	12 28	1 02	12 1	

* Out of cage during night—cold and dehydrated but viable

Histological Specimens (Table 3)

Pup 1—Cells are normal, marrow cells are numerous

Pup 2—Cells are normal, marrow cells are numerous Glycogen is well shown

Pup 3—Cells are normal, marrow cells are numerous

Pup 4—Cells are normal, marrow cells are numerous A few bile canaliculi show brown colloid deposits Lobulation is very well outlined due to contained blood

Pups 305-1, 2, 3, and 4—Cells are normal, but stuffed with glycogen, marrow cells are numerous

Pup 308-1—Cells are normal, but stuffed with glycogen, marrow cells are numerous

Pup 305-5c—Cells are normal, there is no necrosis, marrow cells are numerous A few bile canaliculi contain brown colloid

Pup 305-6c—Cells are normal, there is no conspicuous glycogen no bile canaliculi visible, and the marrow cells are numerous

Pup 305-7c—Cells are normal glycogen is visible and fairly abundant, marrow cells are numerous and there is no necrosis

Pup 308-3c—Cells are normal, there are no glycogen granules, no necrosis, and marrow cells are numerous

Pups 308-4 and 5c—Cells are normal, there are no glycogen granules no necrosis marrow cells are numerous A few bile canaliculi contain brown colloid.

Table 3 shows the nitrogen and sulfur values for pups' livers both normal and exposed to chloroform *in utero*. It is fair to say that in the 48 hour pups, whether exposed to chloroform or not, the N and S values and N/S ratio fall within the range of normal adult dogs. In the pups 7 hours after birth the glycogen deposits are very great but the N/S ratio is unchanged. The amount of N to body weight shows that the pups have livers larger in proportion than adults. Chloroform anesthesia sufficient to kill the mother within 15 to 20 hours causes no abnormality in the pups *in utero* during the anesthesia recognizable by chemical analysis (Table 3) or by histological study.

DISCUSSION

What mechanism accounts for the liver cell necrosis due to chloroform? No adequate explanation has yet been given, but we have suggested that the —SH groups of vital enzyme systems may be concerned (14). We have also speculated on the relation between liver sulfhydryl and the tension of available oxygen, the importance of the available oxygen tension for decreasing chloroform liver damage has been emphasized by the work of Goldschmidt, Ravdin, and Lucké (5). We need not repeat that argument, but may say that the rapid uptake by the depleted liver cell of fed methionine and cystine (Table 2) supports these hypotheses.

What shall we say of the observation that methionine given 3 or 4 hours after chloroform anesthesia gives definite protection to the protein-depleted dog? At first sight this may seem to confuse the issue, but any adequate explanation of chloroform liver necrosis must comprehend this observed fact. We may say that the injury done the liver cell by chloroform during the anesthesia period is in a measure reversible during a 3 or 4 hour period. If a disturbance of an enzyme system is responsible, then that process is reversible during an interval of 3 or 4 hours.

When methionine (a single dose) is fed to a protein-depleted dog, it is retained within the body (17). It does not appear in great concentration in the muscles but does appear in large amounts in the liver (Table 2). We believe the methionine is incorporated in the liver protein matrix just as readily as the same protein matrix was depleted of its sulfur by prolonged low protein diet periods.

Liver proteins are in a state of constant flux as incoming materials are synthesized into protein and out going proteins (e g fibrinogen, prothrombin) are constantly supplied to fill urgent body requirements. There must be some modification of liver protein to produce plasma proteins (fibrinogen) just as plasma proteins must be modified slightly when they supply the protein needs of body cells (21). This implies that liver proteins have a variable make-up—that these proteins are a part of a dynamic system—that certain amino acids may be lost or again regained without serious disturbance of the cell function. There are opinions for and against this argument. In favor are the experiments of Schoenheimer and Rittenberg (16), Schenk and Wollschitt (15), against is the paper of Lee and Lewis (10).

Pups after birth or fetuses *in utero* have an extraordinary tolerance for chloroform anesthesia. After birth the pups slowly lose this peculiar tolerance for chloroform and by the 4th week have reached the level of the adult dog (20). Chemical analyses show no significant differences between pups and normal adults (Tables 2 and 3). It is noteworthy, however, that the N/S ratio of the pups is normal as compared with healthy adults but that the *mothers* showed a very high N/S ratio, that is, *less sulfur*. Apparently the fetuses *in utero* can rob the mother liver of sulfur, as is also true for iron. The needs of the growing fetus take precedence over those of the maternal body—a very important biological law.

One structural difference must be mentioned—the presence of blood islands or *marrow cells* within the liver lobules in the pups. These islands of marrow cells gradually disappear as the pup loses its tolerance for chloroform. This fact is readily demonstrated but adequate explanations to fit this observation are not yet at hand.

The liver lobules of the fetus or pup except for these blood-forming cells are much like the adult liver lobules. The liver lobule of the newborn pup is a little smaller than the adult liver lobule, and the cell nuclei a little larger and more active looking, and more mitoses are to be found.

The pup's liver within 9 months grows to its adult size—an increase of about 20 times. What goes on in the liver lobules meanwhile is not mentioned in the textbooks of anatomy. We get the impression that the formation of new lobules is not accepted but the biliary tree *must* lengthen as the liver size expands. The common duct at the hilum, let us say, is a fixed point and the liver capsule moves out as the liver weight increases. The biliary tree must lengthen its branches and almost certainly the terminal arborizations must increase in number or, in other words, new lobules must be formed. New liver cells must be formed rapidly during growth but these changes apparently have little effect upon chloroform tolerance. The *cross section* of the liver lobules remains relatively unchanged and therefore there is no *structural* basis for the observed tolerance of pups to anoxia.

From the work of Himwich and his collaborators (4, 9) we know that new born pups are highly resistant to the injurious effects of anoxia. This tolerance to anoxia may be a part of the same physiological mechanism which renders these pups so resistant to chloroform anesthesia.

The presence of blood forming cells within the liver remains as one certain structural difference between these fetuses or newborn pups and the adult dogs. However these marrow cells are not uniformly distributed in the liver lobules and one never sees occasional areas of liver cells (free of marrow cells) involved in necrosis and other areas (containing marrow cells) uninvolved in the liver injury. We have only one suggestion to make—that the presence of these marrow cells in some obscure fashion modifies the enzyme system, disturbance of which in the normal adult dog is responsible for the liver necrosis.

As noted above (Table 2) the *protein-depleted dog* is a dog even more completely depleted of sulfur. The maximum differences between the liver N/S ratio of the protein-depleted dog and the normal well fed dog seem too large to be explained solely on the basis of fluctuations in the non protein sulfur content (e.g. glutathione). It appears to connote a definite loss by the protein depleted liver of some relatively sulfur rich component, presumably protein in nature, the loss of which makes the liver more susceptible to a variety of injurious agents known and unknown. When methionine (or cystine) is fed to this type of dog there is a rapid uptake of sulfur, in the liver especially. This response makes up the liver sulfur deficit very promptly. In striking contrast (dog 29-230, Table 1-a, and Table 2) when the methionine is given 6 hours *after the chloroform anesthesia there is no uptake of methionine by the liver*. The liver cells are fatally damaged by the chloroform in this experiment and do not take up the methionine. Obviously this uptake of methionine is not a simple physical response (adsorption) nor is the response related to the Kupffer cells of the liver (reticulo-endothelial system) as these cells are not specifically injured by the chloroform. *Viable liver cells* are essential for the rapid uptake of methionine.

The effects of *protein depletion* on the liver are of general interest not only because of the increased susceptibility to obvious hepatotoxic agents, such as chloroform or arsphenamine (12), but also because of its significant relationship to other disease states—fatty livers (1), experimental cirrhosis (3, 7), experimental liver carcinoma (8), and decreased liver function (18). In almost all of these conditions the beneficial effect of a high protein dietary or more specifically of methionine or cystine or cysteine *plus* choline, points to a fundamentally close relationship between all these abnormal states.

Liver injury has been treated in the past and still is being treated with a high carbohydrate diet. There is good reason for the carbohydrate therapy but, as a result of work in this and other laboratories demonstrating the paramount importance of protein in preventing or allaying liver damage, a diet high in

protein as well as carbohydrate obviously is the correct therapy. The protective action of methionine or cystine plus choline suggests the use of these substances or of suitable methionine-rich protein digests (e.g. casein) as therapeutic agents where any type of liver damage is present. Methionine in solution can be administered parenterally alone or with glucose without any unfavorable reaction in man and animal.

SUMMARY

Protein-depleted dogs are very susceptible to injurious agents—in particular, chloroform. Methionine given shortly *before* chloroform anesthesia will give complete protection against chloroform. Methionine (or cysteine plus choline) given 3 or 4 hours *after* chloroform anesthesia will give significant protection against the liver injury of chloroform anesthesia. Methionine given more than 4 hours after chloroform anesthesia gives no protection against liver injury. Choline alone given before chloroform gives no protection against liver injury.

The protein-depleted dogs have livers which are deficient in both nitrogen and sulfur, but sulfur is depleted more than is the nitrogen. The N/S ratio therefore rises. Methionine or cystine feeding promptly makes up this liver sulfur deficit. Viable liver cells are necessary for this uptake of sulfur.

Livers of fetuses *in utero* or of newborn pups tolerate a chloroform anesthesia which will cause fatal liver injury in adults. The nitrogen and sulfur values of these fetus or pup livers are within the high normal values for adults. *Blood-forming cells* are present in the fetus or pup livers during this period. When these blood islands are eliminated during the 3rd or 4th week of life, the liver then becomes normally susceptible to chloroform liver injury.

Methionine or methionine-rich protein digests (e.g. casein) or various proteins by mouth or by vein should prove useful to protect the liver against certain types of injury and to aid in organ repair.

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SEROLOGICAL REACTIONS OF PROTEIN FILMS AND DENATURED PROTEINS

BY A. ROTHEN PH.D., AND K. LANDSTEINER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The demonstration of compact, globular or ellipsoidal protein molecules by means of Svedberg's ultracentrifuge method (1) in conjunction with diffusion measurements, and, arising from this, the conception of linkages other than peptide bonds which hold the amino acids together in a definite three-dimensional configuration have offered a new problem for serological investigation. While previously proteins were commonly conceived as a linear arrangement of amino acids in peptide linkage, the question now arose whether or to what extent a higher organization of the molecule influences the serological properties of protein antigens, and antibodies. An approach seemed possible through investigations of monomolecular films, where the globular shape is changed into a flat layer, and by consideration of the serological changes occurring in proteins upon denaturation, a process in which a disruption of non-covalent linkages has likewise been assumed (Wu (2), Mirsky and Pauling (3)).

Preliminary experiments along these lines in which monomolecular films of ovalbumin were observed to exhibit specific reactions have been briefly reported by us (4), and the present paper represents an extension of this work.

In addition to papers already quoted there are new reports to be mentioned. Chambers, Bateman and Calkins (5) in a study with spread streptococcal nucleoproteins found that the films were capable of reacting with the corresponding anti-serum. The same workers subsequently investigated the specificity of films of Lancefield's "M" substance (6). They observed that even their thinnest film (14 Å) reacted specifically with homologous sera. The maximum increase in thickness varied from 150 Å for undiluted sera to 50 Å for diluted sera (1/300). The maximum thickness was obtained in 5 minutes for undiluted sera whereas it required 30 minutes to attain the limiting thickness in the case of sera diluted 1/300.

In general the various studies on serological reactions of films have been carried out in part with layers of antigens adsorbed on a metal slide covered with stearic acid and barium stearate in which case the thickness of the layers corresponds at least to the shortest dimension of the globular molecules but often very much thicker layers are obtained (7) and persistence of the original immunological activity may well be expected. In other experiments in which the antigen is first spread on the surface of a buffer solution at a pH near the isoelectric point the thickness of the film is only 6 to 8 Å which corresponds to the average thickness of an extended polypeptide chain. For this reason the use of such monomolecular films, transferred to metal slides coated with stearate is of greater significance for the problem outlined above.

EXPERIMENTAL

Methods—The films were obtained in the following way. A few drops of a 1 per cent solution of the protein to be spread were placed on a piece of filter paper free from any foreign spreading material. The paper was slowly brought into contact with the clean surface of the trough. Two troughs were used, a small one 50×14 cm. made of plastic material (plexiglas) and a large one 90×30 cm. made of enameled brass. Both trays had a well at one end for the transfer of the film to the chromium slides, according to the technique of Blodgett and Langmuir (8). Films were compressed mechanically by chrome-plated brass barriers. The small trough was enclosed in a dust-proof box to avoid contamination through the air. Film pressure was measured by the Wilhelmy balance method as recently described by Harkins and Anderson (9). The sensitivity was of the order of 1 dyne per centimeter of the scale which could be read within a millimeter. The thickness of the films was determined after transfer on chrome-plated slides covered with layers of stearic acid and barium stearate built up in stages ranging from 35 to 51 layers, according to the method of Blodgett and Langmuir (8). The slides had a width of 15 mm. and each of the 10 steps of the optical gauge was 1 mm. wide. The thickness was determined by measuring the angle of incidence at which reflected polarized light (polarized in the plane of incidence) from two adjacent steps appeared of the same intensity. The following equation was used for the calculation

$$t = 24.3 \left[N \frac{\cos r}{\cos r'} - N' \right]$$

where t is the thickness in \AA , N the number of stearic acid layers which gives a minimum intensity in the reflected light for an angle of refraction r , before a protein layer has been deposited. N' is the corresponding number of stearic acid layers after the protein layer or layers have been deposited, the angle of refraction being r' . The constant 24.3 is the thickness of a stearic acid molecule in \AA units as determined by Blodgett and Langmuir.

In the calculation it is assumed (1) that the index of refraction of the protein is the same as that of the underlying stearic acid layers, namely 1.495, and (2) that the phase change occurring at the metal interface does not change appreciably within the range from $i = 67^\circ$ to $i = 85^\circ$ (i = angle of incidence). Both of these assumptions have been shown by Blodgett and Langmuir to be admissible. For ease of computation a table was prepared giving the value of $\cos r$ as a function of i .

The pressure applied to the film for the transfer varied from 2 to 10 dynes. A pressure of 2 dynes was sufficient for the transfer on the "down trip" but at least 7 dynes were necessary on the "up trip" to prevent the layers from slipping back into the trough. Plates coming out of the tray were wet, as is normal with protein films. If the plates were washed before drying, only one layer stayed on. If they were dried first, both layers stayed on, but sometimes the top layer could be washed away. No significant difference was observed in the results, whether a double layer or a single layer was used, in most of the reported experiments single layers were used.

The films obtained with a given protein were not much different in thickness, 6 to 8 \AA for ovalbumin and serum albumin, and 8 to 12 \AA for serum globulin. The thick-

ness was found to be independent of the time during which the films had stayed on the surface showing that unfolding had been completed when the first transfer was made

Tests were made in this manner. After each film had stayed in the trough for a determined period transfers were made to four prepared chrome slides simultaneously and the thickness of the films was measured. A drop of each serum to be tested was deposited on a plate and spread to cover the whole useful area. After an interval of 2 to 5 minutes which was found sufficient for the reaction to go to completion the plates were washed first with saline solution then with water and the increase in thickness measured.

The proteins used were several times recrystallized hen ovalbumin twice recrystallized horse serum albumin and serum globulin which was prepared from horse serum by precipitation between approximately 33 and 50 per cent saturation with ammonium sulfate.

The denatured proteins (ovalbumin horse serum globulin) used for immunization were made in the following manner. A 1 per cent solution in saline was brought in a water bath to 90° and kept at this temperature for 5 minutes. After cooling the coagulated fine flakes were separated washed twice with water and suspended in saline.

For the tests a 1 per cent solution of the proteins in water (not saline) was brought to a pH of about 8.3 and heated in a water bath at 90° for 15 minutes.

Immune sera for the native proteins were prepared by intravenous injections of 2 cc. of about 0.5 per cent solutions on 6 successive days followed by a week's rest. In all two to four such courses were required. For the immunization with denatured proteins 5 cc. of about 2 per cent suspensions were injected intraperitoneally at 6 day intervals four to six such injections were given. The sera were drawn a week after the last injection.

RESULTS

A representative selection of the experiments with films of ovalbumin and horse serum albumin is given in Tables I, II, and III. It is seen that in agreement with results previously obtained, expanded ovalbumin and serum albumin films react specifically with immune sera, the increase in thickness upon treatment with the sera being 30 to 45 Å with homologous sera, against 5 to 14 Å with heterologous immune sera or normal sera. The results were the same whether the protein had been spread on buffer solutions of pH 4.6 or 7.3,¹ or on water. A specific difference between films made with native or denatured ovalbumin was not observed when the films were tested with antisera either to native or heat-denatured ovalbumin. The time elapsed between spreading and transfer onto the plate, up to 18 hours, had no influence upon the results with ovalbumin but some decrease in thickness of the antibody layer taken up occurred in the case of serum albumin. The effect of temperature on the re

¹ At pH 4.6 the second layer was usually removed in the washing

activity of egg albumin films was tested in the following way The box containing the trough was heated electrically The temperature of the bath was

TABLE I

Reaction of a Double Layer of Egg Albumin Spread on Water, with Homologous and Heterologous Immune Sera and with Normal Sera

Thickness of the double layer 15 to 20 Å.

Antiovalbumin immune sera		Normal and heterologous sera	
No	Thickness of adsorbed layer	No	Thickness of adsorbed layer
	Å		Å
1	55, 58	Normal serum 1	7, 9
2	50	" 2	10, 6
3	53, 59	" 3	9
4	49, 51	" 4	12
5	50, 49	" 5	10, 11
6	49, 50	" 6	7, 9
7	42	Anti horse albumin	24
8	56	Anti-horse serum	19, 10, 20
9	49, 52	Anti horse red cells	22
10	52, 56	Anti-ox red cells	10, 19
11	56, 52	Metanilic acid immune serum	12
12	47, 43		
13	41		

TABLE II

Reactions of Monolayers of Ovalbumin and Horse Serum Albumin Spread at pH 7.3 (Veronal Buffer)

Films	Time interval between spreading and transfer	Antiovalbumin sera No						Anti horse albumin sera No				Anti guinea pig blood No 31	Anti human blood No. 36	Anti horse precipitin No 21	Anti horse blood No 19	Anti ox blood No 20	Anti pig blood No 22	Normal sera
		42	43	1	4	11	27	26	28	30	38							
Ovalbumin 5 to 8 Å thick	50 min	33	29	35	—	—	47	—	—	—	—	9	—	12	8	—	—	7
	100 min	33	33	—	36	—	—	—	10, 5	12	5	—	—	11	12	10	—	—
	18 hrs	29	34	39	35	46	35	—	8	9	5	9	—	5	12	8	—	—
Horse serum albumin 5 to 9 Å thick	35 min	—	—	10	—	12	—	36	—	34, 29	34, 30	10	8	—	—	—	—	6
	2 hrs	2	3	—	—	11	—	23	—	29	27	9	12	—	—	—	—	—
	16 hrs	—	3	—	—	8	—	20-30	29-27	22	—	9	—	—	—	—	—	—

85°C and that of the air above the interphase 95°C Films tested after they had been kept at this temperature on the trough for 15 minutes showed no change in the specific properties

Experiments were carried out to see whether species specificity could be demonstrated in the case of closely related ovalbumins. Films of hen ovalbumin (h o) and guinea hen ovalbumin (g o) were tested against antisera for h o, denatured h o and denatured g o.

TABLE III

Reaction of Immune Sera with a Monolayer of Native or Denatured Egg Albumin Spread at pH 4.62 (Acetate Buffer Ionic Strength 0.05)

Thickness of ovalbumin layer 5 to 8 Å. Figures represent thickness in Å units of adsorbed layer

Film	Sera	Time between initial spreading and transfer on slide			
		1 hr	3 hrs.	6 hrs.	24 hrs.
Native egg albumin	Antiovalbumin No 39		34	34	29 29
	Antiovalbumin No 40		34	34 29	37
	Antiovalbumin No 42				22
	Antiovalbumin No 43				36
	Anti-denatured ovalbumin No 44	45 44	42		42 41
	Anti-denatured ovalbumin No 45	46	38		43
	Anti-denatured ovalbumin No 46	39 24			40
	Anti-denatured ovalbumin No 47		39		43 40
	Anti horse albumin No 29	0			
	Anti-horse globulin No. 31	7	5		9
	Anti-horse blood No 19				3,0
	Anti-pig blood No 48				3
Denatured egg albumin	Antiovalbumin No 42	30 22	28	27	27 22 27
	Antiovalbumin No 43		24	25	23 31 27
	Anti-denatured ovalbumin No 44				35 46
	Anti-denatured ovalbumin No 45				32
	Anti-denatured ovalbumin No 46				47 2
	Anti-denatured ovalbumin No 47	36 40			46 37 3
	Anti-denatured horse globulin No 41 ..				5
	Anti horse globulin No 31				10
	Anti-horse albumin No 29		12	9	9 10
	Anti-pig blood No 48				5
	Anti-guinea pig blood No 34		10	10	10

The same increase was observed when an h.o antiserum was brought to react with a film of h o or g o. Also, no difference in thickness increment could be detected when an h o film was tested with denatured anti h o or anti-g o, and the same was true when a g o film was tested with these last two antisera. Dilutions up to 1/150 did not bring out any difference.

Antisera to horse serum and human serum were tested against films of serum albumin from man, horse, and chicken (Table IV). It appears from the table

that the differences between mammalian and chicken albumins were very marked. In the case of horse and human albumin the specificity difference becomes manifest when the immune sera were used in dilution, a specific reaction was still noticeable with serum diluted 1/200 or more.

Tests with films of native horse serum globulin gave results similar to those with the albumins, denatured globulin reacted with sera for denatured but considerably less with antisera to the native protein (Table V). With globulin

TABLE IV

Reactivity of Films of Human and Horse Serum Albumins Spread at pH 6.4 (Veronal Buffer)

The time interval between spreading and transfer was 1 hour except in the lines marked with the symbol * where only 10 minutes was allowed

Antisera for	Dilution in 1 per cent NaCl	Horse albumin films	Human albumin films	Chicken albumin films
Human serum No 50	Undiluted*	22, 28, 13, 13, 22	48, 43, 53, 39, 48	7, 10, 10
	Undiluted	48, 41, 22, 36, 36	48, 34, 36, 33, 32	
	1/5	10, 14	42, 40	
	1/10	3, 10	22, 28, 22	
	1/20	6, 13, 5	21, 27, 25	
	1/50	10, 11, 9	36, 22	
	1/100	7, 9	40, 36	
	1/200	8, 10, 7, 14	31, 28	
	1/500	14, 6	19, 10	
Horse albumin No 38	Undiluted*	39, 34, 43, 36, 40	37, 36, 20, 29, 27	3, 8, 7
	Undiluted	34, 33, 30, 34	29, 19, 31, 14, 32	
	1/5	30, 23, 40, 24, 27	11, 13, 8	
	1/10	29, 29	9, 12	
	1/20	30, 27	8, 5, 12	
	1/50	27, 21	13, 14	
	1/100	21, 31	14, 12	
	1/200	37, 21, 24, 27, 32	3, 10	
	1/500	26, 29	8, 10	

films it was observed that changes still occurred even after the thickness had reached its minimum value. Upon ageing of the native globulin in the trough there was a pronounced decrease in the specific absorption of antibodies for native globulin so that after about 3 hours the specificity was abolished. Similar results were obtained when the sera for native and denatured horse globulin were diluted 1/10 with saline solution. The diminished activity could not be ascribed to contamination in view of the persistence of the activity of ovalbumin films. Films from native or denatured globulin were not inactivated when tested with immune sera for denatured horse globulin, in this case specific adsorption still took place after 48 hours.

This inactivation of native globulin films with time was observed when the films were kept under zero pressure. In the following experiments the influence of pressure was examined (Table VI). The globulin was spread and after 2 minutes, when measurement showed that minimum thickness had been attained, the film was compressed to 20 dynes, it was found to be still active 16 hours later, having been decompressed immediately before transfer. But a 16 hour old globulin film, kept active by compression, deteriorated within a few hours after decompression as if it had not been previously subjected to pressure. Furthermore, compression of inactivated films did not reconstitute the activity.

TABLE VI

Effect of Compression on the Reactivity of Globulin Film with Homologous and Heterologous Immune Sera

Figures are thicknesses of adsorbed protein in Å

Sera	S t *			S t 2 min	Compressed to 25 dynes for 4 hrs		Decompressed for 1½ hrs	S t 2 min	Compressed to 25 dynes for 16 hrs		S t		Compressed to 25 dynes for 3 hrs
	10 min	120 min	210 min		5 min	210 min							
Antiglobulin No 31	31	17	14	30	27	11	29	30	24	14	14		
Antiglobulin No 32	27	—	10	31	34	10	29	31	27	11	17		
Anti-guinea pig No 34	10	7	10,11	9	6	7	7	7	—	—	—		
Anti-chicken No 11	14	—	12	—	6	8	—	6	—	—	—		
Anti guinea pig No 35									7	12	14		
Anti pig No 22									11	12	15		

* St. signifies time between spreading and transfer on the plate.

In the course of the experiments the following observation was made. When after transfer of an antigen film to the metal slide a double layer of stearic acid-stearate was deposited on top of the protein, there was the normal increase of 48 Å in thickness, but the plate came out wet instead of dry. On treatment with a homologous antiserum unexpectedly a specific increase, 30 to 40 Å, was observed, after covering with three double layers of stearic acid, instead of one as above, this effect no longer took place. Since a reaction between antigen and antibody, in spite of interposed layers, would seem improbable, one must consider that perhaps there were discontinuities or uncovered areas in the stearic acid film, or that a displacement had occurred which would bring the protein film above the stearic layer. Further examination of the phenomenon would seem desirable.

Antibody Films—Experiments on monolayers of antibody globulins were carried out with type specific rabbit antibodies against pneumococci Types I and

III. The antibodies were purified essentially according to Heidelberger and Kabat (10), the resulting solutions containing about 60 to 70 per cent specifically precipitable nitrogen. The tests could not be made with the technique used for protein antigens because, as had been already observed by Porter and Pappenheimer (11), absorbed polysaccharides form only a thin layer whose thickness

TABLE VII

Reactions of Specific Polysaccharide with a Monolayer of Pneumococcus Antibodies I and III Spread at pH 7.3 (Veronal Buffer)

Film	Time interval between spreading and transfer	Thickness of adsorbed polysaccharide		Increase in thickness after adsorption of antibodies	
		I	III	I	III
	mins	Å	Å	Å	Å
Antibodies I 8 to 12 Å thick	15	2	—	44	—
	15	—	3	—	12
	25*	—	0	—	15
	40	5	—	45	—
	40	—	7	—	10
	60	2 3	—	47 44	—
	60	—	3 2	—	18 12
	180	4 4	—	25 19	—
	180	—	2 0	—	13 10
	190*	0	—	29	—
	240	2 2	—	15 15	—
	240	—	0,5	—	19 10
	1260	4 4	—	24 24	—
	1260	—	5 2	—	25 19
Antibodies III 8 to 12 Å thick	30	—	3 7	—	60 51
	30	3	—	6	—
	120	—	2 2	—	48 45
	120	5 2	—	10 6	—
	1020	—	0 1	—	60 60
	1020	3 2	—	18 17	—

* Polysaccharide adsorbed 1 month later

could not be reliably measured. Therefore the antibody films, transferred to the slides, were treated for 2 minutes with 1/10 per cent aqueous solutions of one or the other polysaccharide, and afterwards the plates were covered with the antibody solution corresponding to the polysaccharide used.

It is seen from Table VII that films made with pneumococcus antibodies specifically fixed the homologous polysaccharide since the upper (antibody) layer was considerably thicker with the homologous combinations, which may

² We are indebted to Drs A. F. Coca, Walther Goebel and Kenneth Goodner for kindly providing us with pneumococcus antisera and polysaccharides.

be indicated by the type-symbols I I I or III III III, than with the heterologous combinations I III III or III I I where the application of antibody produced only a small increase in thickness. As would be expected in the combinations I III I and III I III, which are omitted from the tabulation, the third layers were likewise thin.

The activity of antibody I diminished gradually with the time elapsed between spreading and transferring to the plates, but films of antibody III retained their activity for at least 18 hours. Inactivation of films of antibody I on metal slides was not apparent even a month after transfer.

Precipitin Reactions with Denatured Proteins—Although denaturation of proteins would not seem to involve profound chemical alteration, it has been established repeatedly that denaturation causes a considerable change in serological properties, with the result that the reaction with immune sera for the corresponding native protein is abolished or greatly reduced, *vice versa*, immune sera for denatured protein give weak or negative reactions with the native antigen. It has further been reported that denaturation is accompanied by a diminution of species specificity or, according to several authors, destruction of the specific serological character of proteins. Since, as mentioned, denaturation is believed to be connected with a disarrangement of the configuration ascribed to coiling of the peptide chains, in which respect denaturation would be related to the formation of films, it seemed advisable to examine the specificity, in the denatured state, of the proteins that were used for studying the reactions of monolayers.

Tests with rabbit immune sera for native (N) and heat-denatured (D) hen ovalbumin gave results essentially confirming those obtained by TenBroeck and Wu (12) and others, namely, that each sort of serum was almost specific for the corresponding antigen. Sera for D ovalbumin gave at most faint traces of precipitation with (high concentrations) N antigen, N immune sera showed reactions of moderate strength with D albumin. This, however, was probably in part at least due to some spontaneous denaturation during storage of the ovalbumin used for immunization because with immune sera obtained by injection of freshly prepared, only once recrystallized ovalbumin the reactions with D antigens were greatly diminished and occurred only in high antigen concentration.

Sera for N horse serum albumin gave distinct reactions with the denatured antigen, the amount of precipitate being 3 to 6 times greater with N than with D serum albumin, whether this is entirely or in part a true cross-reaction has not been investigated. A preparation of D horse serum globulin, too, gave precipitation of moderate strength with antisera for N horse serum globulin, and overlapping reactions occurred with anti-D globulin sera and N globulin.

The species specificity of immune sera prepared with heat-denatured hen ovalbumin was tested by volumetric measurement of the precipitates formed

upon complete reaction with denatured ovalbumin of guinea hen and goose added in successive portions. Seven sera for heat-denatured hen ovalbumin gave the average relative values hen 100, guinea hen 69, goose 36. Comparison with the figures found by Landsteiner and van der Scheer (13) for the reactions

TABLE VIII
Precipitin Tests

Immune sera to	Native hen ovalbumin				Native guinea hen ovalbumin				Denatured hen ovalbumin				Denatured guinea hen ovalbumin			
	1/400	1/2,000	1/10,000	1/50,000	1/400	1/2,000	1/10,000	1/50,000	1/400	1/2,000	1/10,000	1/50,000	1/400	1/2,000	1/10,000	1/50,000
Denatured hen ovalbumin																
Absorbed with denatured guinea hen ovalbumin	0	0	0	0					++	+±	±	f tr	0	0	0	0
	0	0	0	0					++±	++	±	f tr	f tr	0	0	0
Unabsorbed diluted 1/4	0	0	0	0					++	+±	±	0	++	+±	±	0
	0	0	0	0					++±	++	±	0	++±	++	¾	0
Denatured guinea hen ovalbumin																
Absorbed with denatured hen ovalbumin					0	0	0	0	0	0	0	0	+	+±	±	0
					0	0	0	0	0	0	0	0	++	+±	±	0
Unabsorbed diluted 1/4									+±	++	tr	0	+	+±	±	f tr
									++	++	±	0	++±	++	¾	f tr

The immune sera were exhausted with solutions of denatured hen or guinea hen ovalbumin. For the tests 0.2 cc. of absorbed serum was added to 0.05 cc. of antigen dilutions expressed in terms of dry weight. Readings were taken after 1 hour at room temperature (1st line) and after standing overnight in the ice box (2nd line).

of native hen ovalbumin immune sera with the native proteins (hen 100, guinea hen 42, goose 19) indicates some decrease but certainly no abolition of species specificity.³ Similar results were obtained with ovalbumins treated with half concentrated hydrochloric acid for 15 minutes when tested against immune sera to HCl treated hen ovalbumin. Five such sera yielded the average figures hen 100 guinea hen 73, goose 34.

³ It should be mentioned that the antisera to denatured ovalbumin had a distinctly lower antibody content than those produced with the native protein although giving quite satisfactory precipitin reactions.

In absorption tests the preservation of specificity of denatured ovalbumin was strikingly demonstrable (*cf* Mizokuti (14)). Such experiments are presented in Table VIII and show that after exhaustion of a serum for D hen ovalbumin with D guinea hen ovalbumin it still reacted markedly with the former and no longer with the latter—a converse experiment with serum for D guinea hen ovalbumin gave an analogous result. Likewise, in specificity tests with sera for D horse serum globulin and denatured serum globulin of horse, ox, cat, and chicken pronounced species specificity of the denatured proteins was demonstrable.

COMMENT

The specific absorption of antibodies by protein films indicates that structures other than those resulting from the folding of the molecules into compact shape partake in the serological reactivity of proteins. This may be explained by assuming that the constitution of the peptide chain itself furnishes the combining pattern. Another interpretation is that advanced by Pauling (15) (*cf* Wrinch (16)) "that the process of surface denaturation involves the unleafing of the layers without the loss of their structure" (so that the folding in each layer would be like that in the original protein). This concept of a two-dimensional pattern may perhaps seem to be supported by the inactivation of globulin monolayers upon being kept on the water surface. On the other hand, ovalbumin monolayers did not degenerate under the same conditions and there are experiments which indicate that peptide chains as such can suffice as determinants for the reaction with protein antibodies. Pertinent in this connection are specific inhibition reactions of dialysable split products of proteins (17) and the existence of antibodies to fibrous proteins, keratin (18), and silk (19). That after heat denaturation proteins still exhibit very definite species specificity, even if to a somewhat lesser degree than in the native state, is difficult to harmonize, without further theorizing, with the current idea that denaturation goes hand in hand with disruptive breaking of the bonds which hold the folded peptide chain in a fixed configuration, and that it is this structure which is responsible for the specific reactions of the native protein. However, the reason for the marked difference in serological properties between native and denatured proteins, which bears upon this question, has not yet been determined with certainty.

The observations that films made from N and D ovalbumin, supposed in both cases to consist of denatured protein, appeared to react similarly with either N or D antisera, are in contrast to the results of precipitin reactions and therefore call for further investigation.

From the results with antibody films it may be inferred that also the reactivity of antibodies does not necessarily depend upon the "globular" configuration of the molecule. This however, does not exclude an arrangement due to "second-

ary" linkages, in a plane, and it is possible that antibodies exist which on account of a more complex structure would be inactivated by spreading. It should further be mentioned that in experiments of Danielli, Danielli, and Marrack (20) antibody films were found to be inactive. As suggested previously (4), if one may assume a great variety of structures due to folding of the peptide chain this would help to explain that out of the globulin of one species a vast number of different antibodies can be fashioned, very similar if not identical both in chemical composition and antigenically. From other considerations the concept of coiling of peptide chains as basis of antibody structure has been emphasized and elaborated by Pauling (15).

SUMMARY

Films of several proteins, hen ovalbumin, horse and human serum albumins, and globulins were found to combine specifically with antibodies, showing that the reactions can take place independently of the structure which secures the compact shape of the protein molecule. Serum globulin films differed from ovalbumin in that they lost their reactivity when kept on the water surface. Species specific reactions were observed with films of serum albumin and suitably diluted antisera, and likewise in the customary precipitin tests in which immune sera for denatured hen ovalbumin were tested against ovalbumin of other species. Specific fixation was also observed upon exposing monolayers of purified antibodies for pneumococci of Types I and III to the corresponding polysaccharides.

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QUANTITATIVE STUDIES OF THE PHOTOCHEMICAL DESPECIATION OF HORSE SERUM*

AN APPROACH TO THE PROBLEM OF INTRAVENOUS FOREIGN PROTEIN THERAPY

By J P HENRY M.B

(From McGill University Clinic Royal Victoria Hospital, Montreal)

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The antigenicity of foreign protein solutions complicates their employment either as plasma substitutes, or as nutritive agents. Recently interest has been aroused in the possibility of doing away with antigenicity, while retaining the great molecular weight of the protein. The present paper deals with the effects of light energy in the reduction of the antigenicity of horse serum, thus rendering it tolerable to guinea pigs. The problem of sensitivity as a bar to the intravenous injection of foreign proteins is discussed incidentally, and the methods currently employed to reduce antigenicity are considered.

Salter (1) first demonstrated with experimental animals the nutrient value of serum when given parenterally. Among the larger domestic mammalian sera he found normal horse serum the least likely to give rise to immediate symptoms or to that delayed pyrexia, arthropathy and exanthem which was frequent in all animals receiving large amounts of foreign serum. He determined that toxicity is associated with the globulin fraction. By heating the serum he greatly reduced both the immediate and delayed toxic effects. Correlating the available evidence he stated the serum from any given animal is practically innocuous towards other animals of the same species.

Rous and Wilson (2) found early in 1918 that in rabbits, homologous plasma was superior to both acacia and gelatin in restoring and maintaining the circulating fluid volume lost in hemorrhagic shock, and therefore suggested its use in human beings. Later in the year Mann (3) arrived at exactly the same conclusion working with anesthetized dogs submitted to intestinal manipulation. He strongly recommended the use of homologous plasma and suggested its storage for emergencies.

Rous and Wilson (2) noted further that in non-sensitive animals, foreign serum was fully effective in the immediate treatment of shock. They followed the blood pressure in patients given antipneumococcus horse serum for pneumonia and recorded a serious fall in blood pressure on injection of 2 cc. into a sensitive man but no symptoms with amounts as large as 90 cc. in a non sensitive man. They concluded that only in grave emergencies is the use of horse serum justifiable as a plasma substitute. Working on a larger scale, and with bovine in place of horse plasma, Kremen *et al*

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(4) have recently repeated and confirmed these results. It is interesting to note that in England in 1918, Ward (5) unaware of the results of Rous and Wilson, suggested the use of plasma for the treatment of hemorrhage, and proposed comparative studies, similar to those these authors had published. The use of homologous plasma for the combat of shock has followed as a logical sequel to these early studies.

Whipple has for many years studied the metabolic effects of homologous serum given intravenously (6), and his findings, with their emphasis upon the importance of plasma proteins in cellular nutrition, in the protection of the liver, and in resistance to infection, supplied a sound experimental basis for contemporary clinical exploitation of the plasma fraction of blood. Whipple's group attempted to use horse plasma in dogs, but reported that it could not be used owing to toxic symptoms (7). Salter had greater success with heated serum in rabbits, and was able to demonstrate a slight nitrogen sparing effect in some cases (1). Recently Wagensteen *et al* (8) have shown that similar favorable changes in nitrogen balance can be demonstrated with bovine plasma when given to non-sensitive human beings, and Addis (9) has recorded similar results with normal horse serum when given to rats.

Much attention has been paid of late to the isolation of a non-antigenic fraction from the serum of the larger domestic mammals. The comprehensive studies of Doerr and Russ (10 a), Dale and Hartley (11), Hektoen and Welker (12), and Goldsworthy and Rudd (13), have proved that the crystalline serum albumen fraction obtained from horse serum by salting-out methods, has an antigenic specificity distinct from that of the globulins, and is less active in this respect. Hooker (14), and more recently, Janeway and Beeson (15), and Taylor and Keys (16), have demonstrated human sensitivity to non-crystalline foreign albumen fractions.

It is important to determine the degree of reduction of antigenicity that is necessary before a foreign protein solution can be safely used, without regard to the amount injected, or to the sensitivity of the recipient. In considering the use of foreign sera, it is not enough to avoid the toxic effects due to heterolysins and heteroagglutinins, and the discomforts of serum sickness: the problems of sensitization to subsequent doses of the protein, and anaphylaxis in those who are already hypersensitive must also be considered.

With regard to heterolysins and heteroagglutinins, the figures given by Salter (1), Weiss (17), and Uhlenhuth (18) for the relative immediate toxicity of horse and bovine serum have recently been confirmed, in principle, by Davis and Eaton (19). The respective lethal doses per kilo in the rabbit are in the proportion of 5 to 1, i.e., 45 to 9 cc per kilo. Sheep serum lies in an intermediate position. This ratio remains constant in various species, but the dosage in cubic centimeters per kilo varies. In man, the lethal dose of bovine plasma is probably higher. However, Kremen has shown that in spite of treatment by human red cells to adsorb heteroagglutinins, doses as low as 100 to 200 cc gave frequent pyrexial reactions (3). It seems, therefore, that a reduction of such factors to 1/10th to 1/100th their original concentration is desirable.

Gerlough (20) has shown that serum sickness will develop in a percentage of recipients, which varies approximately as the square root of the amount administered. 5 cc of horse serum will lead to symptoms of serum sickness in 10 per cent of the recipients, whence one may conclude that if a litre of protein solution is to be given,

it ought to contain less than this amount of unchanged horse serum, or its equivalent in active antigenicity

Hooker (21) and Park (22) have shown that sensitization will follow remarkably small doses of antigen. Thus a majority of those who had received 1/100 cc. of horse serum years before, were found on skin testing to react positively. A protein solution intended for intravenous therapy in shock, or as a nutritive agent will be given in doses of the order of 1 litre. Therefore in order to avoid any danger of sensitization there must be in 1 litre less than the equivalent in active antigenicity of 1/100 cc. of normal horse serum. That is if the material is obtained from bovine plasma, then the antigenicity of the derivative should be less than 1/100,000th the original. The significance of such a figure is realized on recalling that the albumen obtained by fractionation is probably not less than 1/100th as antigenic as the original serum (10 a)

However, Vaughan (23) has emphasized the difficulties of sensitizing an individual to a protein with which he is constantly in contact. The reason may be, as Walker (24) has shown, that dietary antigens are constantly absorbed unchanged into the blood stream and the evidence from post prandial donor transfusion reactions (23, 25-26) suggests that the amounts thus absorbed and circulating may be quite large, on occasion equivalent in antigenic activity to at least 1/10th to 1/100th cc. of normal horse serum. Vaughan (23) considers that the effect of this continued absorption of foreign protein may be, except in the case of certain allergic subjects to hyposensitize the recipient to these antigens. If bovine serum is employed in place of horse serum (with which little contact is made) there may not be the same dangers of sensitization and shock when a solution is injected which contains small traces of the original antigen.

Hypersensitivity in the frankly allergic may be intense. 1/20th cc. of horse serum injected intradermally in a child weighing 50 pounds has been fatal (27). While it is true that such reactions are rare, and that one fatality occurs to every 50 000 cases treated with serum (22-28) the dangers of spontaneous hypersensitivity are not to be underestimated. For every fatal reaction there will be dozens of dangerous ones and many hundreds of minor disturbances which may prejudice the course of a critical illness. Nor can hypersensitivity be entirely avoided by anamnesis or skin testing. Rutstein *et al.* (29) and Kremen *et al.* (4) have shown that it is possible to obtain very serious reactions in persons who were negative on skin testing, and who gave no history of allergy. On the other hand a litre of fluid is not often given in less than 1 hour and the gradual administration (30) over this period of 1/10th to 1/100th cc. of normal horse serum or its equivalent in active antigenicity will probably not give rise to any uncontrollable reaction in a significant percentage of recipients.

Direction of Approach

If the foregoing analysis is correct, it can be accepted that hypersensitivity is the greatest bar to the use of foreign sera as plasma substitutes. Immediate toxicity, and even serum sickness may possibly be avoided by use of the albumen fraction as suggested by Cohn (31) Davis and Eaton (32) and Keys, Taylor, and Savage (33). Sensitization of normal persons by small doses may perhaps be evaded if the plasma of a food animal is employed. Wolfe (34) Doerr (10 b) and Fleisher and Jones (35),

have shown that the antigenicity of sheep, pig, ox, and horse serum, is of much the same order. Simon (36) has shown that sensitivity to one of these products is accompanied by sensitivity to all of them in over one-third of the positive reactors. It is, therefore, probably not possible to find for all those who are sensitive to one particular protein, another to which they will not react.

An approach to this problem would be the development of a method of removing protein antigenicity, without at the same time reducing molecular weight. The immunological results of four such methods have been studied extensively. Heat, first employed by Salter (1), and later studied by Schmidt (37) and Furth (38), will reduce antigenicity to roughly 1/10th. The use of acidification and alkalinization, as studied by TenBroeck (39), Landsteiner (40), Wells (41), and more recently by Fleisher and Jones (42), and Davis and Eaton (43), will reduce antigenicity, to perhaps, 1/1,000th. The same is to be said of the controlled peptic hydrolysis, first studied by Michaelis (44), and more recently employed by Weil, Parfentjev, and Bowman (45), and later Pope (46), for the purification of antitoxins. Finally, the yeast ferment taka diastase, used by Coghill and Fell (47), would appear to give a similar reduction, based on experimental and clinical study (48, 49).

The effects of these agents upon the physical chemical characteristics of proteins have been studied in both the ultracentrifuge (50, 51) and the Tiselius apparatus (52-54). All give rise to increased dispersion, suggesting marked variations in molecular size, and also, probably in molecular length (75). Under their influence there is an increase of the beta globulin peak, at the expense of the other protein fractions. Similarly, the immunological changes show, in common, a deviation in specificity accompanying the reduction in antigenic activity of the treated product, with the possible exception of peptic digestion. Since there is usually left in such sera, a certain percentage of unchanged protein, a quantitative description of the activity of the mixture involves two estimates. The order of magnitude of the activity per cubic centimeter of the new specificity must be stated in terms of some convenient unit, e.g., normal horse serum. Further, the residual content per cubic centimeter of material having the same activity as the normal unchanged protein must be determined.

It is possible to measure these changes by three methods. First, by use of quantitative anaphylactic studies of the guinea pig *in vivo*, patterned on those employed by Doerr and Russ (10 a) and by Wells (55), in their original studies. They based their estimates on the constancy of the minimal sensitizing dose of horse serum given subcutaneously to a young guinea pig, i.e. 1/100,000 cc. The minimal shocking dose they found to be 1/100 cc. The incubation period, Doerr (10 a) has shown, depends on the dosage of antigen employed. That is, large doses of antigens of low activity, such as albumen, will give rapid sensitization, while small doses of active antigens will require up to 30 days. The minimum sensitizing dose mentioned refers to incubation periods of 2 to 3 weeks. Longer time intervals may decrease the dose required still further. An *in vitro* modification of this technique supplies a second and more sensitive method.

Dale (56) has shown that the isolated uterine horn is also capable of a quantitative response, and that if fully sensitized, it will respond to normal horse serum in concentrations of 1/10,000 to 1/100,000 cc serum per cc bath fluid. The activity of a

solution containing either an unknown quantity of normal horse serum or an antigen of unknown activity, can then be titrated by determining the minimal sensitizing and shocking doses of the material.

A further method is the injection of equal amounts of materials of known and unknown antigenicity into two series of rabbits and the titration to optimal proportions of the resultant precipitins. The general law governing the precipitins developed may be assumed to follow the pattern of that for serum sickness, and to vary as the square root of the antigen dosage. However, this is an assumption, and individual differences between animals are very marked. For these reasons it is not possible to correlate the results of this technique closely with those of the guinea pig studies. By using these three separate methods and by applying them at various stages of any process employed to reduce antigenicity, a reasonable degree of conviction might be attained. Accuracy would be further increased by using large numbers of animals to smooth out individual variations. The quantitative aspects of the following studies were based on this assumption.

While the results are considered accurate to less than one decimal place, the actual reduction required in order to render a foreign serum innocuous is from at least four to six such orders of magnitude. It is as important to give estimates of the reduction attained by any method of despeciation as it is to have a clear conception of the order of reduction required in the first place. Therefore emphasis has been placed throughout upon the quantitative aspect of the findings.

Methods and Materials

Study of the various denaturing agents available led to the choice of light energy. It offered many practical advantages notably simplicity, sterility, possibility of operation at low temperatures, and of ready expansion to large scale production. In addition certain theoretical considerations, to be outlined later, influenced the decision.

It has long been noted (57-58) that ultraviolet light reduces the activity of protein antigens, in the sense that irradiated sera react poorly with antisera to normal serum. However, no studies had been made to determine whether the changes involve merely a deviation in specificity or whether in addition a reduction in antigenic activity occurs. In 1935 Kallos (59) in Upsala, suggested that ultraviolet light might be employed to remove the serum sickness factor from therapeutic sera. Following up this suggestion, Stecher (60) and his coworkers studied the active antigenicity of irradiated serum and found that although specificity had been deviated, antigenicity was if anything, enhanced. They (61) warned against the casual adoption of Kallos' suggestion.

The use of visible light with a photosensitizer has also long been known to produce changes in antigenicity but similarly the active antigenicity of these products have never been studied (62). In the course of his work on photo-oxidation, Smetana at Columbia in 1938 (63) obtained evidence suggesting that photo-oxidised proteins were no longer antigenic, and in 1941 he confirmed the point (64). His statement that 'photo-oxidation does not produce a different antigen but destroys the antigenicity of the protein,' contrasts sharply with Stecher's observations. This diametrical opposition resulting from the use of two methods so closely allied can be resolved if

it is noted that Stecher irradiated whole serum incompletely, allowing it to stand under a lamp for a few hours only. Smetana used pure ovalbumen with a photosensitizer in high concentration in a modified Warburg apparatus. The protein solution was thoroughly agitated for 4 days, and tests for antigenicity undertaken only when photo-oxidation appeared complete. Analysis suggests that his tests would not have detected a reduction in antigenicity below 1/100th the original. It may be that the sequence of events is the same as for other methods, such as alkali treatment. That is, while partial irradiation deviated specificity, prolonged irradiation reduced the activity of this new antigen to a point where Smetana's contention that it was virtually destroyed, was in essence correct. It was decided to attempt to reconcile these two points of view and to explore further the possibilities raised by Kallos of using irradiation as a practical method of reducing the antigenicity of foreign sera.

Methods of Irradiation

The low pressure mercury arcs recently developed for germicidal purposes (65) and now commercially available, were employed as a source of ultraviolet light. They

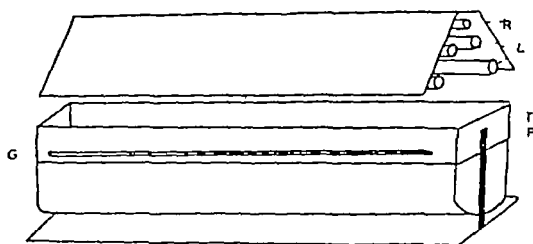


FIG 1 Light bank and tank for irradiation. R, reflector, T, tank, G, glass rod, L, lamps, P, pivot

are efficient, economical, and cool, running at 40°C. Designed to give a maximum output at the wave length desired, their radiation is as nearly monochromatic as is possible without the use of complex apparatus. That is, about 90 per cent of their energy is in the 2537 Å line (65). By using an aluminum reflector, together with five 15 watt General Electric Company¹ lamps, arranged in a "v" bank (Fig 1), an intensity of about 600 microwatts per cm² of energy in the desired 2537 Å line could be applied to the serum to be irradiated. The intensity of irradiation was measured by a standard selenium iron photoelectric photometer to which was attached a zinc silicate fluorescent screen. By this means the approximate constancy of output throughout the course of the experiments was established.

Visible light was obtained from three blue, and two green, fluorescent tubular lamps. Their design was the same as that of the ultraviolet lamps, except that to increase the wave length of the light to the visible range, the inside of the tube was

¹I am indebted to the General Electric Company of Canada for supplying me with data on the output of the lamps employed.

coated with various fluorescent salts, such as zinc silicate (66). Their energy output and spectral distribution were approximately known from published data. Their actual output when in the bank was measured with a calibrated photometer and found to be 700 foot candles. The serum was placed in an enamel tray of dimensions $18 \times 4 \times 2$ inches, under the lamps. The tray itself fitted into a tank which contained a refrigerating coil which provided a means of temperature control. The tank was mounted on pivots and connected by a rod to an eccentric which was driven at some 60 to 100 revolutions per minute by an electric motor. The speed of the motor was controlled by a series resistance. Within the tray was placed a mercury loaded sealed glass tube which moved from side to side as the tray was tilted. By adjusting the speed of the motor and the diameter and loading of the tube, agitation could be made very complete without the development of frothing. The temperature of the irradiated solution did not rise above 25 C. Refrigeration was not employed. The sterility of solutions was assured by autoclaving or Seitz filtering all materials using precautions for asepsis throughout, and by the germicidal effect of the irradiation itself. Since there was some evaporation sterile distilled water was added by a burette connected by a rubber tube to a fine capillary. Bacteriological tests were kindly performed on a sample of irradiated serum by Professor Frederick Smith of the Department of Bacteriology of McGill University and the serum was found to be sterile.

The Serum and Sensitizer—Sterile normal horse serum was obtained through the courtesy of the Parke Davis Company of Detroit. The asepsis employed permitted the omission of preservatives. It was considered important to employ normal horse serum since Doerr (67) has shown that the antigenicity of a serum may increase ten fold during the induction of the hyperglobulinaemia of immunity. Before subjection to irradiation the serum was diluted 1:1 with either 0.85 per cent saline, or with $\kappa/15$ phosphate buffer at pH 7. 150 cc. of the solution were placed in the large tray or 50 cc. in a smaller tray, $8 \times 3 \times 1$ inch thus giving a layer only 3 to 4 mm deep. As a photosensitizer, hematoporphyrin a product of the Nordmark Chemical Company was employed in a 0.2 per cent solution in $\kappa/15$ phosphate buffer. It was sterilized by Seitz filtration.

The Progress of Irradiation—The degree to which the precipitin reaction to anti normal horse serum had decreased was chosen as an index of the progress of irradiation. In order to follow this reduction to the furthest extent possible a high titre antiserum was obtained by giving a prolonged course of immunization to rabbits. This was then titrated against specimens of irradiated sera taken at various time intervals. On some occasions the proportions were held constant at 1:400 on others titration was to optimal proportions. The amounts used were 1/10 cc. of antiserum to 2 cc. of antigen dilution. The containing tubes were shaken, stood for 18 hours at room temperature and the resultant turbidity read in a slightly modified Libby photoreflexometer (68). Thus a quantitative measure could be obtained of degrees of antigen antibody combination so slight that a turbidity was hardly visible and the ring precipitating test indefinite. Calibration with barium sulfate showed that in the region of the opacities employed the galvanometer readings were directly proportional to the amount of material in suspension. Over the range employed the galvanometer readings were thus proportional to the amount of insoluble antigen.

antibody complex formed. A method sensitive to faint turbidity was considered especially desirable under the circumstances of this study, since the aim was to determine the point at which no further reactions occurred. Observations were controlled throughout, either by repetition of results with an antiserum of lower titre, or by running parallel estimates, substituting saline for irradiated serum, and normal rabbit serum for immune anti serum.

The precipitins which developed in a series of twelve rabbits receiving various sera were also followed. Each was given 20 mg, either of normal horse serum, or of irradiated protein, eight times, at 4 day intervals, three times intravenously and five times intraperitoneally, and bled, when fasting, 6 days later. These antisera were not pooled, but titrated separately to optimal proportions, and the ensuing opacities read with the photometer. In some cases a control serum was taken before the course began, in order to demonstrate by direct comparison, any minimal development of precipitin that might have occurred.

To study the changes in antigenicity resulting from irradiation, 180 to 250 gm guinea pigs were sensitized by intraperitoneal injection of 1 mg of protein material, some once, and some three times at 3 day intervals. Young, healthy animals of the same breed were used throughout the studies in order to obtain constancy of reaction and high sensitivity. The diet was kept constant and was of adequate vitamin C content. They were tested at intervals varying from 2 to 4 weeks by intravenous injections and observed for symptoms. The intravenous injections were usually made into the saphenous vein. In a few cases the ear, jugular, or cardiac route was employed. Immediately after injection the animal was liberated, his behavior noted, and his rectal temperature followed as an index of the severity of a reaction (10c). Autopsy was always carried out, and any cases receiving cardiac puncture observed for hemopericardium.

The Dale uterine horn technique was carried out in a smaller muscle bath than usual, 1 c, 2 cc. The calcium content of the Dale's solution was reduced to one half in accordance with Dale's original observation (56) that spontaneous contractions were thereby reduced. Increasing concentrations of the test antigen were added, and the bath emptied and refilled between each addition until full anaphylaxis had occurred. The completeness of the reaction was confirmed by failure to respond to a second dose in spite of a full response to a standard dose of 0.5 gamma histamine per cc. This constant dose of histamine also served as a standard by which the responses of two differing uteri could be compared.

In order to study roughly the chemical changes developed, the xanthoproteic Millon's, and Hopkins-Cole color reactions (69) were applied to the sera and to their dialysates. The effects of the various standard protein precipitants were noted, the pH changes observed roughly with nitrazine paper, and the histamine content estimated by the method of Barsoum and Gaddum (70). The total nitrogen, and also the nitrogen not precipitable by phosphotungstic acid was estimated by the micro Kjeldahl technique.

RESULTS

Irradiation with Visible Light—The effects, under varying conditions, of a series of irradiations with visible light were followed by use of the precipitin

reaction at constant proportions Fig 2 shows the results when diluted horse serum was irradiated with blue green light of 600 foot candles both with and without hematoporphyrin Curve 1 shows how slight are the changes to be obtained with the use of light alone, and curve 2 the effects of prolonged agitation in the dark To do this, 5 cc of serum were placed in a 4×1 inch vial and rolled in the tray for 72 hours In curve 3 is seen the powerful effect

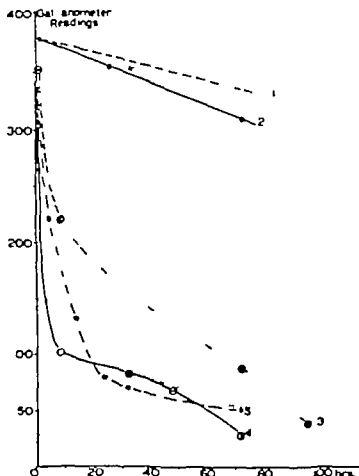


FIG 2 Titration with 0.1 cc. of antiserum at constant proportions of 2 cc. of 1:400 dilution of irradiated sera Curve 1 600 foot candles, blue green light without sensitizer Curve 2 agitation in the dark Curve 3 600 foot candles blue green light and 2 mg of hematoporphyrin per 100 cc A further 4 mg added at 72 hours. Curve 4 same light but 20 mg hematoporphyrin added per 100 cc and a further 20 mg added at 32 hours. Curve 5 same light but 4 mg hematoporphyrin added every 6 hours for 24 hours

of the addition of 2 mg of hematoporphyrin per 100 cc. and the additional effect when at 72 hours 2 more mg were added Curve 4 demonstrates the effects with ten times the amount of hematoporphyrin. In this case the second dose was added at 32 hours. Curve 5 demonstrates that the addition of 16 mg of hematoporphyrin in four 6 hourly doses, has, if anything, a slightly greater effect than the larger single initial dose of 20 mg

Since curve 4 showed the closest approach to extinction of the precipitin

TABLE I
Anaphylaxis of Guinea Pig to Serum A

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
NHS, 0.1 cc. given 3 times at 3 day intervals	21	Serum A 2 cc	—	2 guinea pigs injected
Serum A, 0.2 cc given 3 times	21	NHS 2 cc NHS 1 cc	++++ +++	1 pig injected 1 " "
Serum A, 0.2 cc given once	27	Serum A 0.2 cc 2 cc	++++ ++++	1 " " 1 " "

NHS indicates normal horse serum

Death, +++++, severe anaphylaxis, +++, dubious symptoms, ±, unaffected, —

TABLE II
Minimal Lethal Shocking Dose of Horse Serum for Sensitized Guinea Pigs

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
NHS, 0.1 cc given 3 times at 3 day intervals	28	NHS 1.5 cc " 0.2 cc. " 0.02 cc. " 0.05 cc.	++++ ++++ ++++ ++++	3 pigs injected 3 " " 1 pig injected 1 " " (Autopsy confirmatory on all 8 pigs)
" "	28	" 0.01 cc	++	1 pig Temperature constant
" "	28	" "	+++	1 pig Temperature 101-96°F
" "	28	" 0.005 cc	+++	1 pig
" "	28	" 0.002 cc.	+	2 pigs Temperature constant
NHS, 0.1 cc given once	22	" 1.0 cc " 0.5 cc " 0.1 cc " 0.01 cc	++++ ++++ ++++ ++++	1 pig 2 pigs 3 " 2 " (Autopsy confirmatory on all 8 pigs)

reaction, the irradiated material, which may be termed serum A, was employed to sensitize six guinea pigs. In Table I it is seen that 2 cc of the 1:1 dilution with normal saline, containing 10 mg of irradiated protein, failed to cause anaphylaxis in an animal fully sensitized to normal horse serum. Specificity has thus been deviated. That animals sensitized with serum A react to normal horse serum,⁸ would seem to confirm the results of the precipitin test, and show that sufficient unchanged horse serum antigens remain in the 3 mg of serum A to sensitize to horse serum. The reaction of serum A sensitized animals when given serum A, proves that not only is specificity deviated, but the material is strongly antigenic.

In Table II are presented the control data obtained with guinea pigs sensitized with the same batch of normal serum. These animals developed fatal

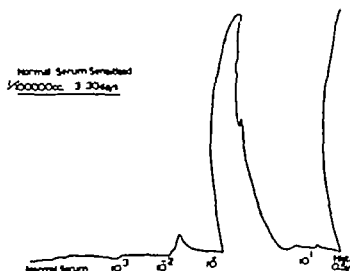


FIG 3 Dale test with a guinea pig uterus sensitized to normal serum. The animal sensitized with 3 injections of 1/100 000 cc. of normal horse serum at 3 day intervals and tests made 30 days after the last injection.

shock with a minimal dose in the region of 1/100 cc., and symptoms with about 1/500 cc. Thus, the sensitivity of the animals was the same as those studied by Doerr (10a) and by Wells (55). The minimum sensitizing dose is the same as that found by these authors, *i e*, approximately 1/100,000 cc., as may be seen in the accompanying uterine tracing. In the case of Fig 3 the animal received a sensitizing dose of three injections of 1/100,000 cc. of normal horse serum 4 weeks before testing.

Rough quantitative estimates of these changes can therefore be made. Since 1/500 cc of normal serum will usually cause symptoms in a normal serum sensitive animal, the now altered proteins in 2 cc. of serum A were equivalent in antigenic activity to less than 1/100 cc. of the original unchanged horse serum per cc. The fact that 1/5 cc. of serum A killed serum A sensitized pigs suggests that the fresh antigenicity of serum A was at least 1/20th that of

normal horse serum, if the minimum lethal dose of normal horse serum is accepted as 1/100 cc

Irradiation with Ultraviolet Light—Diluted sera were irradiated with approximately 400 microwatts per cm^2 of 2537 Å light from three 15 watt lamps. Fig. 4 shows the results of the study of the precipitin reaction at constant proportions. Curve 1 was determined for serum with 20 mg of hematoporphyrin added, that had been irradiated with only the visible and long ultraviolet

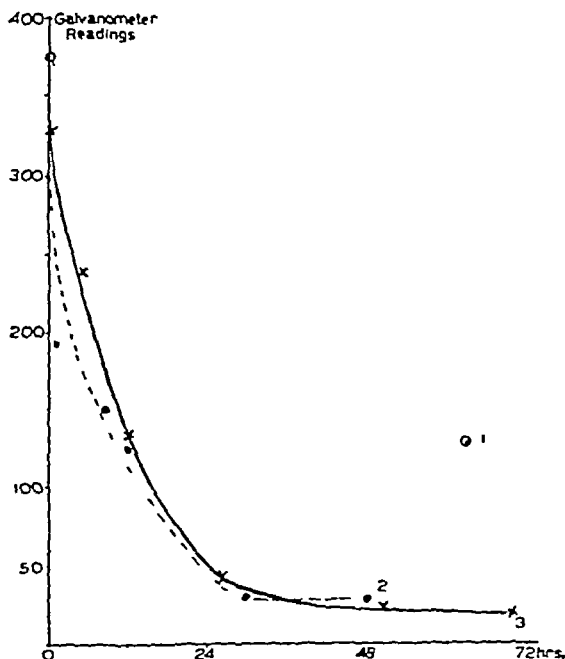


FIG. 4 Effects of ultraviolet light with and without sensitizer. Titration of 0.1 cc high titre rabbit antiserum with 2 cc of 1:400 dilutions of irradiated sera. Curve 1, visible light output only of ultraviolet lamps serum sensitized with 20 mg hematoporphyrin per 100 cc. Curve 2, 400 microwatts per cm^2 2537 Å ultraviolet (serum B). Curve 3, 400 microwatts per cm^2 ultraviolet with 20 mg hematoporphyrin per 100 cc.

output of these lamps, the short ultraviolet being cut off by a plate glass filter. There is as marked a reduction in precipitin reaction in 72 hours as is obtained with the whole output in 14 hours. This shows that the light from the lamps is by no means all confined to the 2537 Å line. Curve 2 is for ultraviolet light alone without a sensitizer, and curve 3, that for ultraviolet light with 20 mg of hematoporphyrin per 100 cc. Curve 3 suggests a slightly increased effect as the result of this addition.

To determine more accurately the changes occurring, a series of curves were derived from titration to optimal proportions of sera irradiated with 600 microwatts per cm^2 of ultraviolet light for varying periods. Fig 5 shows the progressive changes in precipitin reaction up to 96 hours irradiation. This curve was derived from serum that had been irradiated for 96 hours with an initial addition of 20 mg of hematoporphyrin per 100 cc. The parts of the 48, 72, and 96 hour curves in antigen concentration above $1/128$ were corrected for

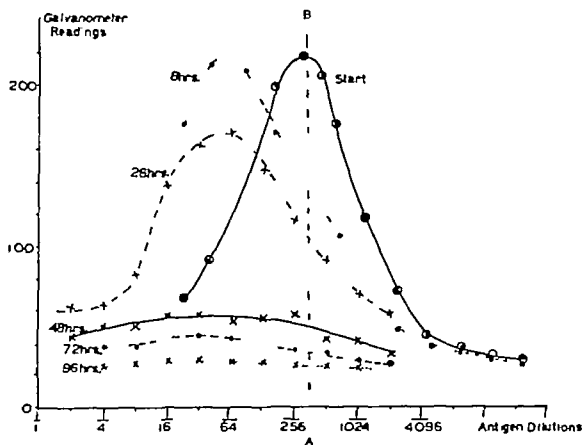


FIG 5 Effects of irradiation of horse serum antigens with ultraviolet light. Titration to optimal proportions of 0.1 cc. horse antiserum with various dilutions of horse serum. Start before irradiation. 8 hours, after 8 hours 600 microwatts per cm^2 light of 2537 \AA . 26 hours after 26 hours of same irradiation. 48 hours, after 48 hours 72 hours after 72 hours 96 hours after 96 hours of 600 microwatts per cm^2 of ultraviolet light of 2537 \AA with addition of 20 mg of hematoporphyrin per 100 cc.

the opalescence that develops in the more irradiated sera. This was accomplished by reference to the control titrations with normal rabbit serum which were set up in parallel with every titration against the anti horse immune rabbit serum.

The physical characteristics of the serum which had been irradiated for 72 hours with 600 microwatts per cm^2 of ultraviolet light without photosensitizer, and which may be termed serum B, were as follows. It was of the

same color, but had become slightly opalescent. There was a very faint odor of burnt protein. The protein precipitants, e g, trichloroacetic acid, phosphotungstic acid, and salts of the heavy metals, produced voluminous precipitates. Unlike normal serum, the addition of 0.2 cc normal acetic acid in equal volume caused a heavy precipitation, but on the other hand, boiling failed to cause any turbidity or precipitation. Dialysis through No. 450 cellophane against normal saline gave a dialysate which became turbid on

TABLE III
Anaphylaxis of Guinea Pig to Serum B

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
Serum B, 0.2 cc given 3 times at 3 day intervals	22	Serum B 2 cc	++++	1 pig Autopsy confirmatory
" "	22	0.7 cc	+++	1 pig Temperature 100-96°F
" "	22	0.2 cc	+++	1 pig Temperature 101-99°F
" "	22	NHS 2 cc	++++	1 pig Autopsy confirmatory
" "	22	" 0.4 cc	++++	1 pig Autopsy confirmatory
" "	22	Serum B 2 cc	++++	1 pig Autopsy confirmatory
" "	22	" " 2 cc	+	1 pig Temperature constant
Serum B, 0.2 cc given once	22	" " 2 cc	+	1 pig Temperature constant
" "	22	" " 1.5 cc	-	3 guinea pigs
" "	22	NHS 1 cc	+	2 " "
NHS, 0.1 cc given 3 times	22	Serum B 2 cc	-	2 " "
NHS, 0.1 cc. given once	22	" " 2 cc	-	3 " "
		" " 5 cc	-	1 " pig

Death, +++++, severe anaphylaxis, +++, definite, +, dubious, ±, unaffected, -

adding tannic acid or heavy metals. The color tests for the benzene ring and for tyrosine were as strongly positive for the irradiated protein as for normal serum. That for tryptophane however was only doubtfully present. Intermediate stages in irradiation showed diminishing amounts of tryptophane. None of the tyrosine present in the dialysate was free, it was all incorporated in that material in the dialysate which was precipitable by tannic acid or by heavy metals. To control the possibility of adsorption, free tyrosine was added to the dialysate and was readily detectable.

Tests at intervals with nitrazine paper showed that unless the serum was buffered, the pH fell progressively during irradiation, from 7.0 to 4.5 at 72 hours. The histamine content increased from the normal value of 0.004 gamma per cc. only to 0.0080 gamma per cc. The total nitrogen content of the original serum was 11.7 mg per 100 cc., and of this, 0.7 mg was not precipitable by phosphotungstic acid. After irradiation, this fraction rose to 2.1 mg per cc., showing that 1.4 mg of the original 11.7 mg, or about 10 per cent, had been reduced to a form not precipitable by phosphotungstic acid.

In Table III are seen the summarized results of studies with serum B upon the guinea pig *in vivo*. Animals sensitized to normal horse serum do not react to serum B in amounts up to 5 cc. A single injection of 0.2 cc. of serum B



FIG. 6 Dale test with control non-sensitized guinea pigs.

does not sensitize, either to normal serum, or to serum B. On the other hand, three injections of 0.2 cc. sensitize both to normal serum and to serum B, and fatal shock was obtained with the latter with a dose of 2 cc., but not with a dose of 0.7 cc.

These results suggest that the proteins in 1 cc. of serum B are equivalent in antigenic activity to less than 1/5,000 cc of normal horse serum, for serum B fails to shock normal serum sensitized animals, and 0.2 cc. fails to sensitize to normal horse serum (the minimal sensitizing dose of normal serum is of the order of 0.00001 cc). Since it requires more than 1 cc of serum B to shock fatally, serum B sensitized animals, the actual powers of shocking, haptenic powers of serum B are approximately 1/100th those of normal horse serum (Table III). The active antigenic powers of serum B may be even less than

this, since one dose, 1,000 fold the sensitizing dose of normal horse serum fails to sensitize to serum B

In the Dale test, the non-sensitized uterus did not respond to concentrations of normal serum and of serum B up to 1:100 in the bath (Fig 6). The normal serum sensitive uterus reacted to 1:100,000 of normal serum, but 10,000 times

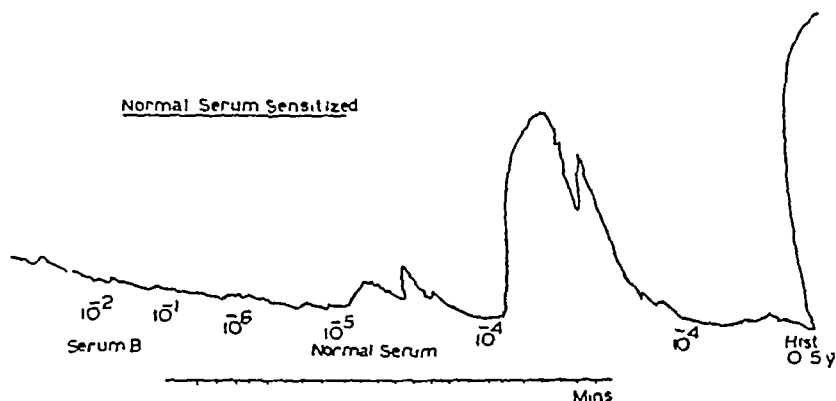


FIG 7 Dale test with guinea pigs sensitized to normal serum. Sensitized with three injections of 1/10 cc normal horse serum at 3 day intervals, and tested 3 weeks after the last injection

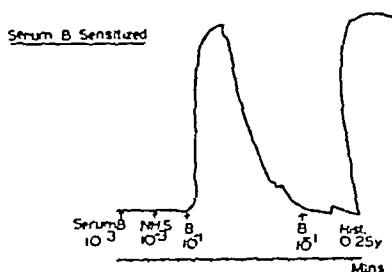


FIG 8 Dale test with guinea pigs sensitized with serum B. 200 gm pigs given 10 mg protein intraperitoneally three times, at 3 day intervals, and tested 3 weeks after the last injection

this concentration of Serum B failed to elicit a response (Fig 7). In contrast, a three times serum B sensitized guinea pig uterus did not react to serum B until a concentration between 1:100 and 1:10 was reached (Fig 8). These tracings, confirmed by three others not illustrated, support preceding approximations that the antigenic activity of serum B was reduced to 1:10,000 in terms of normal horse serum, and that the new active antigenicity of deviated

specificity of this serum is of the order of 1/1,000th that of normal horse serum

Fig 9 represents the average of the values of the individual titration of the four antisera to normal serum obtained after giving the standard course to

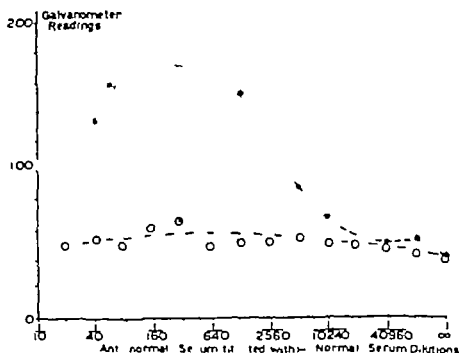


FIG 9 Antiserum to normal serum Circles control titration of normal rabbit serum with normal horse serum Dots rabbit antisera to normal horse serum, titrated to optimal proportions with normal horse serum

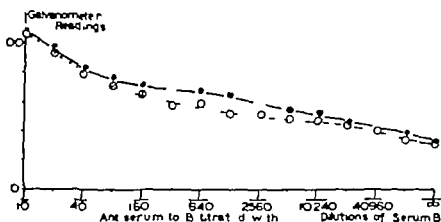


FIG 10 Antisera to serum B Circles, control titration with normal rabbit serum Dots rabbit antisera to serum B titrated to optimal proportions 0.1 cc. with 2 cc. of varying dilutions of serum B (Average of five curves)

four control rabbits. The contrast with Fig 10 is sharp. Here the averaged results of equivalent courses of serum B given five rabbits are shown. On averaging the titrations of the five antisera to serum B with normal serum, there was no detectable reaction, showing that any antibodies developed are

TABLE IV
Anaphylaxis of Guinea Pig to Serum C

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
NHS, 0.1 cc given 3 times at 3 day intervals	27	Serum C 2 cc	—	(1) 300 gm pig
Serum C 0.2 cc.	19	" " 2 cc	—	(3) 800 " pigs
" "	26	" " 2 cc.	—	(3) 200 " "
" "	"	" " 4 cc	±	(1) 200 " pig Temperature 100–102°F
" "	19	NHS 2 cc.	—	(3) 800 gm pigs
" "	27	" 1 cc.	+	(1) 180 " pig
" "	27	" 2 cc.	+	(1) 240 " "

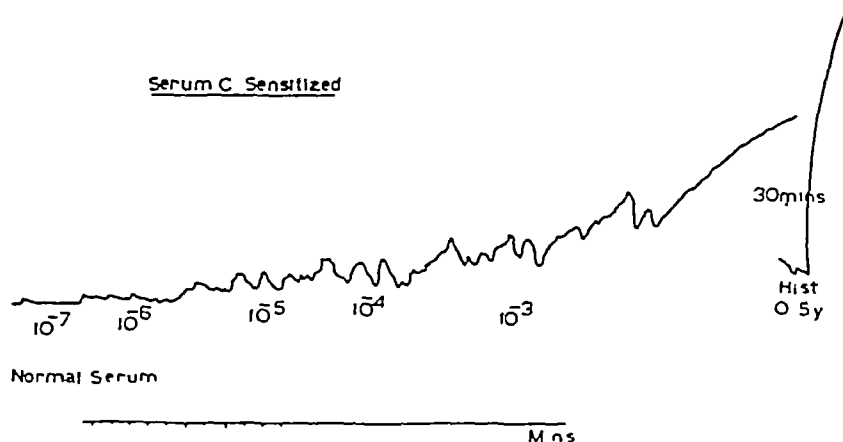


FIG 11

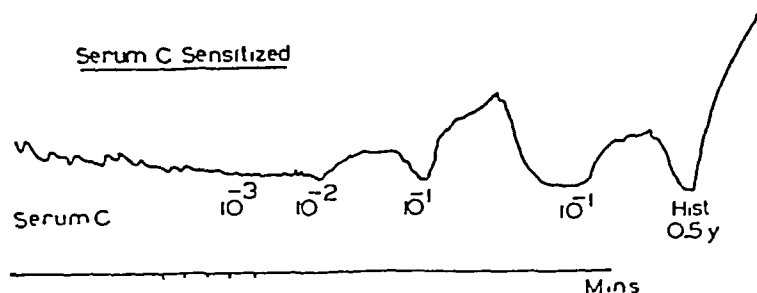


FIG 12

FIGS 11 and 12 Dale test with guinea pigs sensitized with serum C 200 gm guinea pigs given 10 mg protein intraperitoneally three times at 3 day intervals, and tested 4 weeks after the last injection

to the altered antigens in serum B, and that if any minute quantities of normal serum antigens do remain, they have given rise to no observable antibody concentration

To find whether these results could be confirmed, serum was irradiated for 96 hours with 600 microwatts of ultraviolet light in the 2537 Å region. It was buffered with $\pi/15$ phosphate to pH 7 and treated with 20 mg of hematoporphyrin per 100 cc. In this product the test for tryptophane was negative. It was expected that the antigenic activity of this serum, termed serum C, would be even lower than that of serum B. Table IV shows that in spite of three

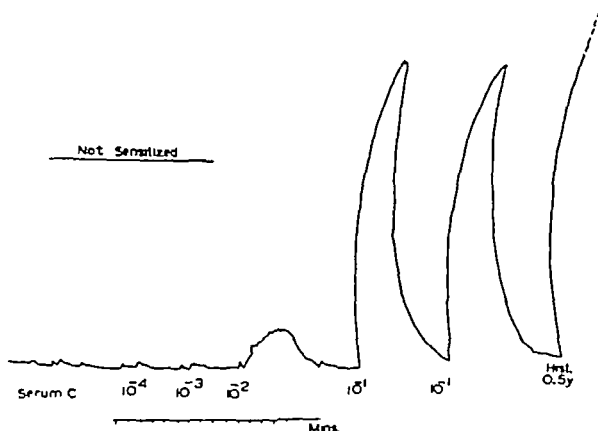


FIG. 13 Dale test with serum C upon the normal guinea pig uterus

intraperitoneal injections of 1 mg. of protein, and an incubation period of 3 to 4 weeks, a shocking dose of up to 4 cc. of serum C failed to elicit symptoms. Nor were these animals affected by what would be in those sensitized to normal horse serum, two hundred times the minimal lethal dose of normal serum. The implication following the reasoning already employed is that the active antigenicity of serum C is of the order of 1/10,000th that of normal serum, and that the residual content of antigens having the specificity of normal horse serum may be as low as the equivalent of 1/100th cc. of horse serum per litre.

The uterine strip which is more sensitive than the tests in vivo confirms these results. Animals sensitized with three injections of 0.1 cc. of serum C reacted

after an incubation period of 1 month, very incompletely to doses one hundred times the full shocking value (Fig 11). This suggests that in the three doses of 1/10th cc, there was less than the antigenic equivalent of 1/100,000 cc of normal serum (Fig 12), and demonstrates that in serum C sensitized uteri, a reaction occurs only at high concentrations of serum C. Since it can be repeated, and since it also occurs in the non-sensitized uterus, (Fig 13) it is not anaphylactic, but may be due to the effect of break-down products. On titrating three antisera to serum C and averaging the results, a slight but definite reaction was observed when the graph was compared with the average

TABLE V
Effects of Irradiation upon the Antigenicity of Normal Horse Serum

	Normal serum	Serum A	Serum B	Serum C
Treatment	—	600 ft. candles blue green 72 hrs with 20 mg Hp per 100 cc	600 micro- watts per cm ² 2537 Å 72 hrs	600 micro- watts per cm ² 2537 Å 96 hrs with 20 mg Hp per 100 cc
pH	7.0		4.5	
Tyrosine	++++		++++	
Tryptophane	++++		±	—
Histamine	0.004 gamma per cc		0.008 gamma per cc	
Approximate active anti- genicity	1	$\frac{1}{20}$	$\frac{1}{1,000-5,000}$	$\frac{1}{5-20,000}$
Residual unchanged horse serum antigenic equivalents, cc/cc	1	$\frac{1}{1,000 \text{ cc}}$	$\frac{1}{10,000 \text{ cc}}$	$\frac{1}{100,000 \text{ cc}}$

Hp, hematomorphyrin

of the control titrations of serum C, with the sera taken before the immunising courses were started. As in the case of serum B, no antibody to normal horse serum was detected in the antisera to serum C. The reactions in all but one of these cases were so indefinite that they could be detected only with the photometer.

A summary of the more significant findings appears in Table V. The numbers of animals used, the variety of the techniques, and the controls employed throughout, serve to counterbalance the inaccuracies of the methods, and random errors of individual variations. Therefore, approximate values have been affixed for the order of magnitude of the antigenicity of the materials

studied, for without such an estimate it is not possible to assess their activity relative to each other, and to those materials, such as horse serum, whose effect upon human beings is now well known.

DISCUSSION

The masterly studies of Rideal and Mitchell (71*a*) upon the photochemistry of the peptide link have shown that in surface films the chief effect of light of energy content from 3000 Å to 2600 Å is to rupture those links in the polypeptide chain immediately adjacent to the aromatic residue. The photochemical threshold for this process lies at 3000 Å and is also the threshold for absorption by the aromatic nucleus (71*b*). The benzene ring will not resonate to incident photons of a longer wave length. The energy of combination of the CO—NH link, is approximately one-fifth that of a photon of threshold wave length. If a means of transmitting the energy of photons of longer wave length were available it would still be sufficient to rupture the peptide links. Photosensitizing dyes probably supply structures which, resonating to these longer wave lengths perform this transmission of energy (72, 73).

Sanigar, Krejci, and Kraemer (74) studying the effects of ultraviolet light upon human serum albumen have obtained evidence suggesting the splitting off of low molecular weight fractions of high aromatic amino acid content. At the same time, their ultra-centrifugal studies suggest that the majority of the protein increases in size as a result of aggregation. The average resulting molecular weight they estimate as between two to three times the original. The protein is highly polydisperse, with probable marked variations in molecular length (75) as well as in weight. These findings have been confirmed by Svedberg and Brohult (76). Bernhart (77) using dialysis and nitrogen estimates as a gauge of molecular weight changes has found marked increases in the dialysable low molecular weight fractions following prolonged ultraviolet irradiation.

The physical chemical findings after irradiation with visible light and a photosensitizer are similar to those with ultraviolet light. Smetana and Shemin (64) have observed aggregation and polydispersion in studies with the ultracentrifuge. Harris (78), Lieben (79) and Carter (80) have shown that the aromatic nucleus in the protein is selectively affected and Lieben (79) has demonstrated a selective loss in tryptophane and tyrosine. Recently Smetana (64) has confirmed this loss of tryptophane, but not that of tyrosine. During photolysis both by ultraviolet and by visible light, there is oxidation of sulfhydryl groups (81).

The findings with serum B and serum C showed a destruction of tryptophane and dialysable fractions containing aromatic amino acids were also demonstrable. The opalescence obtained suggests the presence of some aggregates of very high molecular weight. Possibly this is to be anticipated in view of the marked polydispersion.

The effects of ultraviolet and of visible light upon proteins are of interest, for here is an agency which strikes directly at the aromatic rings. The greater susceptibility to light of antibodies, as compared with antigens, may be, because in order to function as an antibody, a protein must preserve exactly that pattern which enables it to fit, or neutralize, an antigen. On the other hand,

an antibody so denatured that it is stretched out (82*a*, *b*) and otherwise modified so that it could not possibly function as an antibody, may still be highly antigenic, albeit with a deviated specificity. There are over seventy tyrosine radicles in a globulin molecule (83), arranged, according to Bergmann's hypothesis (84), in repeating sequence. If light energy disrupts the linkages holding these into the peptide backbone, varying stages of photolysis may be expected to give varying degrees of disorganization of the original pattern. The changes would not necessarily involve either liberation of the aromatic residue by the unlikely event of rupture of the link on each side, or a total photolytic destruction of the residue itself. Landsteiner (85) has emphasized that alkali-treated protein of reduced antigenicity could be made more antigenic by nitration of the aromatic residues. Since the addition of groups to the aromatic residues restores antigenicity, it is not unreasonable to venture that distortion from their normal relations may lead to a great diminution in antigenicity (86, 87).

The evidence presented has shown that under the conditions used, the photo-oxidation of serum with visible light will lead to a deviation in specificity, but to little diminution in antigenicity. Smetana has noted only a loss of antigenicity, however he was using pure ovalbumen and only studied the final stages of photo-oxidation. His evidence does not preclude a deviation in specificity during the course of the process.

The results with ultraviolet light point to a very marked diminution in the original horse serum active antigenicity. The group of curves in Fig 5 are of interest in this respect. The early stages which show a progressive diminution in antigenic activity may be explained as associated with a shift in the constitution of the antigen-antibody complexes from the usual preponderance of antibody to one of antigen (88). It is interesting that after 8 hours there is a persistence of the optimal ratio at approximately constant proportions, and a decrease in the total precipitate, regardless of the amount of antigen added. Smetana's first published curve ((64), Fig 1) with a pure antigen and the single antibody evoked by it, though not carried to such high antigen concentrations as were the data for Fig 5, suggests a similar constancy of optimal proportions.

Haurowitz (89) has shown that several antibodies are elicited to even a single pure antigen. It may be permissible to postulate that even in so simple a system as irradiated ovalbumen/anti normal ovalbumen, irradiation may have had the effect of distorting the antigen molecules so that a decreasing percentage of the variously shaped molecules of the antibody complex will react with them. This might lead to the effect observed, of a steadily decreasing volume of precipitate at approximately constant optimal proportions.

Stecher's (60) conclusion that deviation of specificity follows ultraviolet irradiation is fully confirmed by the studies described. The viscosity of the solutions increased during irradiation. Neurath (90) has shown that changes

in viscosity of protein solutions can be directly related to the degree of extension of the molecule that occurs during denaturation. Thus there is in direct evidence of a change in molecular shape which may be associated with the deviation in specificity observed. The marked decrease in active antigenicity was as definite as the deviation in specificity. This may prove of significance in the problem of removing protein antigenicity.

It is not possible to estimate what part the addition of hematoporphyrin played in the further reduction in antigenicity noted with serum C, since the duration of irradiation was also increased to 96 hours. Harris (78) considered it probable that hematoporphyrin sensitized, not only to visible, but to ultra violet light as well. Since his ultraviolet light was not free from longer wave lengths, proof was not clear. The decrease in precipitin reaction noted in Fig 4, curve 1, with the plate glass filtered light from the ultraviolet lamps, may have been due in part to long ultraviolet waves of the 3600 Å to 3300 Å resonance bands, as well as to the even longer, visible wave lengths produced by these lamps. The effect is sufficient to prevent any clear cut decision that the hematoporphyrin increased the efficiency of the ultraviolet light.

The use of the purified albumen fraction of animal sera as a blood substitute has recently received much attention. If, as has been suggested by Taylor and Keys (16), the use of this fraction in large amounts in human beings is not practicable owing to its residual specific antigenicity, then some method of removing protein antigenicity would become necessary. Such a method could be applied either to serum or to the albumen fraction, as Davis and Eaton (43) have recently suggested, or even to other proteins such as those in milk.

Svedberg (91) has emphasized the value of the photon as a means of applying small and known amounts of energy to proteins and so obtaining a controlled manipulation of these complex delicate molecules. Stimulated by this and other observations, a study of the effects upon proteins of radiations in the range 6000 Å to 2537 Å has been commenced. It is felt that the first results have been sufficiently promising to suggest a careful consideration of this agent as a means of removing protein antigenicity.

Studies of the toxicity of irradiated protein solutions, and of their capacity to substitute for homologous plasma are in progress.

SUMMARY

1 Normal horse serum was irradiated for periods of 3 to 4 days, with visible light or with ultraviolet light of known intensity and wave length. The photosensitizer hematoporphyrin was employed in some instances. The serum was exposed to the air in thin layers, and thoroughly agitated throughout irradiation.

2 The irradiated sera were unchanged in color, and over 90 per cent of the original protein content remained precipitable by phosphotungstic acid.

3 Studies of the antigenicity of the sera were carried out on guinea pigs

an antibody so denatured that it is stretched out (82 *a, b*) and otherwise modified so that it could not possibly function as an antibody, may still be highly antigenic, albeit with a deviated specificity. There are over seventy tyrosine radicles in a globulin molecule (83), arranged, according to Bergmann's hypothesis (84), in repeating sequence. If light energy disrupts the linkages holding these into the peptide backbone, varying stages of photolysis may be expected to give varying degrees of disorganization of the original pattern. The changes would not necessarily involve either liberation of the aromatic residue by the unlikely event of rupture of the link on each side, or a total photolytic destruction of the residue itself. Landsteiner (85) has emphasized that alkali-treated protein of reduced antigenicity could be made more antigenic by nitration of the aromatic residues. Since the addition of groups to the aromatic residues restores antigenicity, it is not unreasonable to venture that distortion from their normal relations may lead to a great diminution in antigenicity (86, 87).

The evidence presented has shown that under the conditions used, the photo-oxidation of serum with visible light will lead to a deviation in specificity, but to little diminution in antigenicity. Smetana has noted only a loss of antigenicity, however he was using pure ovalbumen and only studied the final stages of photo-oxidation. His evidence does not preclude a deviation in specificity during the course of the process.

The results with ultraviolet light point to a very marked diminution in the original horse serum active antigenicity. The group of curves in Fig 5 are of interest in this respect. The early stages which show a progressive diminution in antigenic activity may be explained as associated with a shift in the constitution of the antigen-antibody complexes from the usual preponderance of antibody to one of antigen (88). It is interesting that after 8 hours there is a persistence of the optimal ratio at approximately constant proportions, and a decrease in the total precipitate, regardless of the amount of antigen added. Smetana's first published curve ((64), Fig 1) with a pure antigen and the single antibody evoked by it, though not carried to such high antigen concentrations as were the data for Fig 5, suggests a similar constancy of optimal proportions.

Haurowitz (89) has shown that several antibodies are elicited to even a single pure antigen. It may be permissible to postulate that even in so simple a system as irradiated ovalbumen/anti normal ovalbumen, irradiation may have had the effect of distorting the antigen molecules so that a decreasing percentage of the variously shaped molecules of the antibody complex will react with them. This might lead to the effect observed, of a steadily decreasing volume of precipitate at approximately constant optimal proportions.

Stecher's (60) conclusion that deviation of specificity follows ultraviolet irradiation is fully confirmed by the studies described. The viscosity of the solutions increased during irradiation. Neurath (90) has shown that changes

in viscosity of protein solutions can be directly related to the degree of extension of the molecule that occurs during denaturation. Thus there is in direct evidence of a change in molecular shape which may be associated with the deviation in specificity observed. The marked decrease in active antigenicity was as definite as the deviation in specificity. This may prove of significance in the problem of removing protein antigenicity.

It is not possible to estimate what part the addition of hematoporphyrin played in the further reduction in antigenicity noted with serum C, since the duration of irradiation was also increased to 96 hours. Harris (78) considered it probable that hematoporphyrin sensitized, not only to visible, but to ultra violet light as well. Since his ultraviolet light was not free from longer wave lengths, proof was not clear. The decrease in precipitin reaction noted in Fig 4, curve 1, with the plate glass filtered light from the ultraviolet lamps, may have been due in part to long ultraviolet waves of the 3600 Å to 3300 Å resonance bands, as well as to the even longer, visible wave lengths produced by these lamps. The effect is sufficient to prevent any clear cut decision that the hematoporphyrin increased the efficiency of the ultraviolet light.

The use of the purified albumen fraction of animal sera as a blood substitute has recently received much attention. If, as has been suggested by Taylor and Keys (16), the use of this fraction in large amounts in human beings is not practicable owing to its residual specific antigenicity, then some method of removing protein antigenicity would become necessary. Such a method could be applied either to serum or to the albumen fraction, as Davis and Eaton (43) have recently suggested, or even to other proteins such as those in milk.

Svedberg (91) has emphasized the value of the photon as a means of applying small and known amounts of energy to proteins and so obtaining a controlled manipulation of these complex delicate molecules. Stimulated by this and other observations, a study of the effects upon proteins of radiations in the range 6000 Å to 2537 Å has been commenced. It is felt that the first results have been sufficiently promising to suggest a careful consideration of this agent as a means of removing protein antigenicity.

Studies of the toxicity of irradiated protein solutions, and of their capacity to substitute for homologous plasma are in progress.

SUMMARY

- 1 Normal horse serum was irradiated for periods of 3 to 4 days, with visible light or with ultraviolet light of known intensity and wave length. The photosensitizer hematoporphyrin was employed in some instances. The serum was exposed to the air in thin layers, and thoroughly agitated throughout irradiation.

- 2 The irradiated sera were unchanged in color, and over 90 per cent of the original protein content remained precipitable by phosphotungstic acid.

- 3 Studies of the antigenicity of the sera were carried out on guinea pigs

and rabbits. Fresh antigenicities of deviated specificity and of an activity of the order of 1/50th, 1/1,000th, and less than 1/20,000th that of normal horse serum were obtained. The residual content of material having the same antigenic specificity as normal horse serum was estimated as approximately equivalent in activity to dilutions of normal horse serum of 1 cc, 1/10 cc, and less than 1/100 cc per litre respectively.

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THE IMMUNIZATION OF FOWLS AGAINST MOSQUITO BORNE PLASMODIUM GALLINACEUM BY INJECTIONS OF SERUM AND OF INACTIVATED HOMOLOGOUS SPOROZOITES*

By PAUL F RUSSELL, M.D., AND B N MOHAN

(From the Pasteur Institute of Southern India Coonoor, India)

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That immunity in malaria is dependent on the combined interaction of both cellular and humoral agencies now seems clear Cannon and Tahaferro (1931), Tahaferro and Cannon (1936), Tahaferro and Mulligan (1937), among others, proved the importance of the lymphoid macrophage system in defence against malaria Coggeshall and Kumm (1937, 1938), Mosna (1938), and others have presented proof of the existence of protective antibodies in the blood of monkeys with a high degree of acquired immunity Manwell and Goldstein (1938, 1940), Hegner and Dobler (1939), Tahaferro and Tahaferro (1940), and others have shown that similar protective humoral antibodies occur in avian malaria Mulligan *et al* (1940) were the first to demonstrate clearly the effects of correlated interaction of both cellular and humoral agencies in simian malaria Their observations suggested that the enhanced value of the immune serum they used in treating *Plasmodium knowlesi* infections was directly correlated with the degree of "stimulation" of the lymphoid macrophage system caused by previous infection with *P cynomolgi* Their results seemed to indicate that cellular and humoral agencies in malarial defence were so closely interdependent that, under the conditions of their experiments, a full measure of one was relatively ineffective in the absence of an adequate measure of the other

In the first experiments of our series (Mulligan *et al*, 1941, Russell *et al*, 1942) we found that repeated injections of large numbers of inactivated sporozoites of *P gallinaceum* into domestic fowls brought about a partial immunization against mosquito-borne infection with the homologous *Plasmodium* This paper reports not only more experiments of this sort but also experiments in which serum instead of inactivated sporozoites was used, and finally experiments in which both were used to test the effect of combined cellular and humoral defence stimulation

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Materials and Methods

Materials and methods have already been described (Russell *et al* , 1942) and any exceptions or additions will be noted as indicated below. Each of the infections reported in this paper was caused by the bites of two infective mosquitoes. This yielded a fairly large infective dose of sporozoites which was employed because we have found that occasionally no infection in a susceptible fowl will follow the bite of one mosquito though on subsequent dissection there are seemingly normal sporozoites in its salivary glands. This may be due to occasional abnormal blocking of the salivary duct of the mosquito, though we have not been able to prove this. In all cases the infecting mosquitoes were dissected to prove the presence of sporozoites in salivary glands.

Mosquitoes were incubated at about 80°F with about 80 per cent relative humidity for development of sporozoites.

Mortality among experimental fowls due to intercurrent diseases was a serious problem. For example, the 69 previously unpublished experimental fowls listed in Tables II, VII, and XII were the balance from 144 with which the experiments were started. In other words, 52.1 per cent of our birds died either before infection by mosquito or during the prepatent period thereafter. Except in the cases of fowls 349 and B 97 any fowl dying with a positive blood smear was considered to have died from malaria. In the cases of 349 and B 97 signs of the virus disease called "drooping sickness" were unmistakable. The percentage of red cells infected when No. 349 died was only 12 and in B 97 it was less than 1 per cent. No control fowl in the entire series died with a percentage of red cells infected of less than 21. Therefore, Nos. 349 and B 97 were classed as casualties prior to completion of experiment. Control fowls naturally were much less affected, and only four, or 5.5 per cent, of 73 died of intercurrent disease. The combined mortality rate for both experimental and control animals from intercurrent disease was 36.4 per cent.

We gave much time and attention to this difficulty, practising strict quarantine and scrupulous cleanliness, using pyrethrum sprays and powder for insects. The fowls were purchased locally and were of decidedly poor quality. It would undoubtedly have been much wiser to have incubated our own supply, not only to avoid the cholera, influenza, pox, virus diseases, etc., which afflicted the fowls, but also for greater uniformity of experimental animal.

Some deaths were due to anaphylactic shock, some to embolism, and perhaps some to infection introduced with dried ground thoraces of mosquitoes, but the majority died of recognizable infectious fowl diseases.

Results of Sporozoite Vaccination

In Table I are shown data from a series of 10 fowls (A 2, 4, 5-7, 11-14, 22) which received sporozoite vaccination only. Each fowl received five intravenous injections of a saline suspension of ground dried thoraces of infective mosquitoes, between July 23 and August 18, the numbers of mosquitoes used for each fowl totalling 220. In each case the agglutinating titre of fowl serum for homologous sporozoites was tested and in no case was it below 1/65,536 dilution. Each fowl was infected by the bites of two infective *Aedes albopictus*

mosquitoes, 15 days after these insects had taken their infective blood meal. Five control birds (A 36-40) were bitten by two mosquitoes from the same lot, and one (A 41) by two mosquitoes 25 days after their infective meal.

TABLE I

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Sporozoite Vaccine Third Series

Fowl No.	Weight of fowl gm	Agglutinating titre of serum	Prepatent period days	Percentage of red blood cells infected																		Result
				Days following infection																		
				8	9	10	11	12	13	14	15	16	17	18	19	20	21	22				
Experimental																						
A 2	777	65 536	8	N	+	+	4	5	1	+	N	+	N	N								SR
A 4	733	131 072	8	N	+	+	7	25	60	63	D											D
A 5	584	131 072	9	N	N	+	3	10	27	41	D											D
A 6	779	131 072	8	N	+	+	6	13	13	7	+	N	N									SR
A 7	688	131 072	9	N	N	+	+	1	4	2	N	N										SR
A 11	743	65 536	10	N	N	N	+	4	4	7	8	4	+	+	+	+	N	N				SR
A 12	873	131 072	8	N	+	+	5	4	3	3	+	N	N									SR
A 13	670	131 072	10	N	N	N	+	+	4	3	+	+	N	N								SR
A 14	774	131 072	8	N	+	+	6	18	60	D												D
A 22	673	65 536	10	N	N	N	+	+	2	+	+	N	N									SR
Control																						
A 36	728	—	9	N	N	+	3	8	58	61	D											D
A 37	730	—	8	N	+	+	5	15	36	51	30	29	52	72	92	D						D
A 38	653	—	8	N	+	+	4	16	33	D												D
A 39	676	—	8	N	+	+	2	9	14	24	62	D										D
A 40	560	—	9	N	N	+	2	6	2	1	+	+	+	+	N	N						SR
A 41	838	—	14	N	N	N	N	N	N	N	+	3	+	+	N	+	N	N				SR

Average prepatent period 8.8 days experimental fowls, 9.3 days controls.

" highest infections 20.5 per cent experimental fowls, 42.8 per cent controls.

Mortality rate 30.0

66.7

Each fowl bitten by two mosquitoes 15 days after their infective meal (25 days in case of A 41).

N = negative smear D = died of malaria SR = spontaneous recovery + = positive smear but less than 1 per cent of red cells infected. Counted in totals as 1 per cent.

It will be seen (Table I) that the average incubation periods were 8.8 and 9.3 days in experimental and control fowls, respectively. The mortality rates were 30.0 and 66.7 per cent, respectively. Parasite counts were made and the percentages of red blood cells infected are shown by daily count in the table. The average of the highest percentages was 20.5 in the experimental birds and 42.8 in the controls.

In Table II are grouped together a total of 19 fowls which were infected after sporozoite vaccination. The first two series have already been published (Mulligan *et al*, 1941, Russell *et al*, 1942). It will be seen that the average incubation period for the combined series was 8.9 days, the average mortality 21.1 per cent and the average of highest percentages of red cells infected was 20.5 (only the last series counted in this way). These data may be compared with those for normal or control fowls shown in Table III. Here we have combined 11 series, the first two of which have already been published (Mulligan *et al*, 1941, Russell *et al*, 1942). The combined total of normal, *i.e.*,

TABLE II

Mosquito Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Sporozoite Vaccine Three Series Combined

Series No	Serial Nos. of fowls	No. of fowls in series	Deaths from malaria	Spontaneous recoveries	Mortality rate	Prepatent period		Highest percentages of red cells infected		Remarks
						Total	Average for series	Totals	Average for series	
						days	days			
1	127, 150	2	0	2	Nil	17	8.5	—	—	Previously published*
2	165, 166, 171, 173, 176, 177, 185	7	1	6	14.3	64	9.1	—	—	" "†
3	A 2, A 4-7, A 11-14, A 22	10	3	7	30.0	88	8.8	205	20.6	See Table I
Totals		19	4	15	21.1	169	8.9			

Each fowl bitten by two infective mosquitoes

* First series published Mulligan *et al*, 1941

† Second " " Russell *et al*, 1942

untreated fowls was 83, and among these the average prepatent period was 9.1 days, the average mortality 55.4 per cent, and the average of highest percentages of red cells infected 30.1 (three series, as shown, not counted in this way). The sporozoite vaccination apparently produced a partial immunity.

It is not entirely clear why the last series of vaccinated fowls (A 2, 4, 5-7, 11-14, 22) had a higher mortality than the first 9 birds but we suspect it was because the mosquitoes used were younger. We have now found that generally there is a greater degree of infection caused in fowls by mosquitoes 15 days or less after their infective feed than by those used 19 days or more afterwards. In the earlier series we generally used older mosquitoes.

TABLE III

Plasmodium gallinaceum Infections in Untreated Fowls Each Bitten by Two Infective Mosquitoes
Eleven Series Combined

Series No.	Serial Nos. of fowls	No of fowls in series	Deaths from malaria	Spontaneous recoveries	Mortality rate	Prepatent period		Highest percentage of red cells infected		Remarks
						Total	Average for series	Totals	Average for series	
					per cent	days	days			
1	61 117 134 158-60 164	7	5	2	71.4	72	10.3	—	—	Previously published*
2	195-198 215 219 220	7	2	5	28.6	60	8.6	—	—	†
3	319-328	10	3	7	30.0	88	8.8	254	25.4	Mosquitoes fed 19 to 25 days after infective feed
4	395-400	6	2	4	33.3	59	9.8	178	29.7	Mosquitoes fed 19 to 25 days after infective feed
5	413-424	12	1	11	8.3	117	9.8	242	20.2	Mosquitoes fed 19 to 25 days after infective feed
6	A 36-A 41	6	4	2	66.7	56	9.3	257	42.8	Mosquitoes fed 15 days after infective feed
7	A 93-A 98	6	3	3	50.0	47	7.8	199	33.2	Mosquitoes fed 15 days after infective feed
8	B 33-46	14	14	0	100.0	126	9.0	—	—	Mosquitoes fed 16 days after infective feed
9	B 70 72, 73 75	4	4	0	100.0	39	9.8	165	41.2	Mosquitoes fed 11 days after infective feed
10	B 76-81	6	6	0	100.0	54	9.0	250	41.7	Mosquitoes fed 11 days after infective feed
11	D 1, 2 4-6	5	2	3	40.0	41	8.2	112	22.4	Mosquitoes fed 11 to 12 days after infective feed
Totals		83	46	37	55.4	759	9.1	1657	30.1	

* Mulligan *et al*, 1941

† Russell *et al* 1942.

‡ Excluding series 1, 2, and 8.

Results of Serum Treatment

In Tables IV to VI are shown data regarding fowls receiving prophylactic serum treatment. The first series (359, 360, 362, and 368) received seven daily intraperitoneal injections of 1 cc normal sheep serum, from June 23 to 30, and were bitten by two infective *A. albopictus* mosquitoes, 19 to 25 days after their infective feed, on July 1 (Table IV). Agglutination tests were made and no titre higher than 1/256 dilution was found. Six control birds (395-400) were used and each was bitten by two mosquitoes of the same lot used for the

TABLE IV

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Normal Sheep Serum First Series of Serum Treatments

Fowl No	Agglutinating titre of fowl serum	Prepatent period	Percentage of red blood cells infected																										Result	
			Days following infection																											
			8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		34
		days																												
Experimental																														
359	128	10	N	N	N	N	+	+	5	3	3	1	+	+	N	N												S R		
360	128	12	N	N	N	N	N	+	+	+	+	+	+	+	N	N												S R		
362	256	8	N	+	+	+	3	4	2	+	+	+	+	+	N	N												S R		
368	256	9	N	N	+	+	5	15	14	42	30	15	12	5	4	1	+	+	3	9	13	15	28	19	10	3	+	N	N	S R
Control																														
395	—	8	N	+	+	+	5	11	7	+	+	+	+	+	N	N												S R		
396	—	13	N	N	N	N	N	+	+	1	5	33	50	45	39	21	14	3	+	+	3	4	8	14	64	84	D		D	
397	—	8	N	+	+	+	1	10	14	19	24	D																D		
398	—	12	N	N	N	N	N	+	+	+	9	22	39	29	25	7	+	+	+	N	N							S R		
399	—	9	N	N	+	+	4	11	6	+	N	N																S R		
400	—	9	N	N	+	+	6	9	3	+	+	N	N															S R		

Average prepatent period 9.8 days experimental fowls, 9.8 days controls

" highest infections 12.8 per cent experimental fowls, 29.7 per cent controls

Mortality rate nil " " " " 33.3 " " "

Each fowl bitten by two mosquitoes 19 to 25 days after their infective meal

N = negative smear, D = died of malaria, S R = spontaneous recovery, + = positive smear but less than 1 per cent of red cells infected. Counted in totals as 1 per cent

experimental birds. The prepatent period averaged 9.8 days in both experimental and control fowls. The mortality rate in the former was nil and in the latter 33.3 per cent. The average of highest percentages of red cells infected was 12.8 in the experimental and 29.7 in the control fowls.

Data from the second series (A 90, 91, B 64-69) are shown in Table V. Each fowl received seven daily intraperitoneal injections of 1 cc of serum pooled from a number of fowls having chronic malaria due to the homologous *Plasmodium*. Each was bitten by two infective *A. albopictus* mosquitoes 11 days after their infective meal. These mosquito feedings were made after the fourth

injection of serum so that each fowl received 3 cc. of serum after infection. It will be seen (Table V) that the average prepatent period was 8.2 days and in 4 control birds (B 70, 72, 73, 75) it was 9.8 days. The mortality was 25 per cent in experimental and 100 per cent in the control fowls. The average of

TABLE V

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Chronic Fowl Serum. Second Series of Serum Treatments

Fowl No.	Weight of fowl	Pre-patent period	Percentage of red blood cells infected																								Result	Remarks
			Days following infection																									
			7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24								
	gm	days																										
<i>Experimental</i>																												
A 90	530	7	N	+	+	1	6	20	36	43	49	D														D		
A 91	665	8	N	N	+	+	2	9	11	16	14	1	N	N												SR		
B 64	733	8	N	N	+	+	5	9	2	+	+	+	+	N	N											SR		
B 65	839	8	N	N	+	+	1	2	+	N	N															SR		
B 66	894	9	N	N	N	+	+	2	4	9	10	8	6	1	+	+	+	+	N	N						SR		
B 67	1140	8	N	N	+	+	2	5	4	+	+	N	N													SR		
B 68	778	8	N	N	+	+	+	3	3	5	6	4	+	+	N	N										SR		
B 69	775	10	N	N	N	N	+	+	3	5	8	D														D	A virus disease may have been a complication in B 69	
<i>Control</i>																												
B 70	718	12	N	N	N	N	N	N	+	+	1	10	20	31	D											D		
B 72	694	8	N	N	+	+	5	21	D																	D		
B 73	822	10	N	N	N	N	+	+	+	6	12	25	45	41	45	49	52	67	58	D						D		
B 75	642	9	N	N	N	+	+	3	12	31	20	32	46	D												D		

Average prepatent period 8.2 days experimental fowls, 9.8 days control.

Average highest infections 13.1 per cent experimental fowls 41.2 per cent controls

Mortality rate 25.0 " 100.0 "

Each fowl bitten by two mosquitoes 11 days after their infective meal.

N = negative smear D = died of malaria SR = spontaneous recovery + = positive smear but less than 1 per cent of red cells infected. Counted as 1 per cent in totals.

highest percentages of red cells infected was 13.1 in the former and 41.2 in the latter

Data from the third series (B 85, 91, 93, 94, 96, 100) are shown in Table VI. These fowls each received seven daily intraperitoneal injections of 1 cc. normal sheep serum between November 18 and 24. Each was bitten by two *Aedes*

albopictus mosquitoes on November 26, 11 to 12 days after these insects had taken their infective meal. There were five controls (D 1, 2, 4-6) each bitten by two mosquitoes of the same lot. The average prepatent period was 9.2 days in the experimental and 8.2 in the control fowls. The mortality rate in the former was 16.7 per cent and it was 40.0 per cent in the latter. The average of highest percentages of red cells infected was 27.8 in the experimental birds and 22.4 in the controls.

TABLE VI

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Normal Sheep Serum. Third Series of Serum Treatments

Fowl No	Weight of owl	Prepatent period	Percentage of red blood cells infected																		Result
			Days following infection																		
			7	8	9	10	11	12	13	14	15	16	17	18	19	20					
	gm	days																			
<i>Experimental</i>																					
B 85	720	10	N	N	N	N	+	+	3	5	14	21	4	+	N	N			S R		
B 91	626	8	N	N	+	1	6	17	36	32	14	5	+	+	N	N			S R		
B 93	620	8	N	N	+	7	12	3	+	N	N								S R		
B 94	636	11	N	N	N	N	N	+	+	13	24	52	78	D					D		
B 96	950	9	N	N	N	+	+	13	15	4	+	N	N						S R		
B 100	786	9	N	N	N	+	+	4	5	2	+	N	N						S R		
<i>Control</i>																					
D 1	636	8	N	N	+	+	9	23	D										D		
D 2	661	8	N	N	+	+	6	6	7	3	+	N	N						S R		
D 4	657	8	N	N	+	+	+	4	7	27	56	46	42	35	29	D			D		
D 5	664	9	N	N	N	+	+	4	3	1	+	+	N	N					S R		
D 6	686	8	N	N	+	+	5	14	22	8	4	+	+	N	N				S R		

Average prepatent period 9.2 days experimental fowls, 8.2 days controls

" highest infections 27.8 per cent experimental fowls, 22.4 per cent controls

Mortality rate 16.7 " " " " 40.0 " " "

Each fowl bitten by two mosquitoes 11 to 12 days after their infective meal

N = negative smear, D = died of malaria, S R = spontaneous recovery, + = positive smear but less than 1 per cent of red cells infected. Counted as 1 per cent in totals

In Table VII we have combined the above three series of fowls receiving serum treatment. It will be seen that the average prepatent period was 8.9 days, the average mortality 16.7 per cent, and the average of highest percentages of red cells infected 17.9 per cent. These data may be compared with those for vaccinated fowls in Table II and for normal fowls in Table III. It will be seen that average prepatent period and mortality rate were 8.9 days and 21.1 per cent for combined series of vaccinated fowls, compared with figures of 9.0 days and 55.4 per cent for normal fowls.

If the fowls receiving sheep serum are grouped there is a total of 10, with an average incubation period of 9.4 days a mortality of 10.0 per cent, and an average of highest percentages of red cells infected of 21.8 per cent. There were 8 birds which received chronic fowl serum and the corresponding data are 8.2 days' average incubation period, 25.0 per cent mortality, and 13.1 per cent average of highest percentages of infected red cells. The numbers are too small to make much of these somewhat contradictory findings.

Results of Combined Serum Treatment and Sporozoite Vaccination

In Tables VIII to XI are shown data for three series of fowls which were given combined prophylactic serum and sporozoite vaccination.

TABLE VII

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Serum Injections Three Series Combined

Series No	Serial Nos. of fowls	No of fowls in series	Deaths from malaria	Sporozoite recovery	Mortality rate	Prepatent period		Highest per centages of red cells infected		Remarks
						Total	Average for series	Totals	Average for series	
						days	days			
1	359 360 362 368	4	0	4	Nil	39	9.8	51	12.8	See Table IV
2	A 90 A 91 B 64-69	8	2	6	25.0	66	8.2	103	13.1	V
3	B 85, 91 93 94 96 100	6	1	5	16.7	55	9.2	167	27.8	VI
Totals.		18	3	15	16.7	160	8.9	323	17.9	

Table VIII presents data for the first series (231, 233-239, 241, 272). These fowls each received five intravenous injections of saline suspensions of ground dried thoraces of infective mosquitoes between April 17 and May 12, the number of mosquitoes used for each fowl totalling 270. Each fowl also received seven daily intraperitoneal injections of 1 cc normal sheep serum from May 10 to 16. On May 19 each was bled for agglutination test and each was bitten by two infective mosquitoes, probably but not certainly from 15 to 20 days after the insects had had their infective blood meal. (Probably but not certainly *Aedes albopictus*. Any exceptions were *Armigeres oblongatus*.) There were 10 control birds (319-328) each bitten by two infective mosquitoes from the same lot.

It will be seen (Table VIII) that the average prepatent period was 8.2 days in the experimental animals and 8.8 days in the controls. The mortality rate

was nil in the protected fowls and 30.0 per cent in the controls. The average of highest percentages of parasite infected red cells was 18.3 in the former and 25.4 in the latter.

TABLE VIII

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Serum and Also of Sporozoite Vaccine First Series of Combined Treatments

Fowl No	Agglutinating titre of fowl serum	Pre-patent period	Percentage of red blood cells infected																								Result
			Days following infection																								
			7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26					
		days																									
<i>Experimental</i>																											
231	131,072	8	N	N	+	3	13	6	2	+	N	+	N	+	N	+	+	N	N								SR
233	65,536	9	N	N	N	+	2	6	9	7	1	N	N														SR
234	65,536	8	N	N	+	+	1	5	6	3	+	+	N	N													SR
235	262,144	10	N	N	N	N	+	3	11	5	2	N	N														SR
236	131,072	8	N	N	N	+	4	20	30	8	2	+	+	N	N												SR
237	262,144	8	N	N	+	+	4	5	3	+	N	N															SR
238	131,072	7	N	+	+	6	9	16	2	+	N	N															SR
239	131,072	9	N	N	N	+	2	6	32	38	44	28	16	10	5	4					N	N					SR
241	65,536	7	N	+	11	18	26	25	2	7	+	N	N														SR
272	65,536	8	N	N	+	5	23	17	4	N	N																SR
<i>Control</i>																											
319	—	10	N	N	N	N	+	+	5	14	34	35	D														D
320	—	9	N	N	N	+	2	18	17	2	N	N															SR
321	—	9	N	N	N	+	1	6	11	6	1	N	N														SR
322	—	10	N	N	N	N	+	+	7	17	51	59	61	82	D												D
323	—	8	N	N	+	+	12	6	3	+	N	N															SR
324	—	7	N	+	10	40	56	48	27	D																	D
325	—	9	N	N	N	+	+	5	16	19	6	+	N	N													SR
326	—	10	N	N	N	N	+	5	4	3	2	+	+	+	N	N											SR
327	—	8	N	N	+	1	3	4	+	+	3	2	+	+	+	N	N										SR
328	—	8	N	N	+	N	+	+	4	12	5	3	+	+	+	N	N										SR

Average prepatent period 8.2 days experimental fowls, 8.8 days controls

" highest infections 18.3 per cent experimental fowls, 25.4 per cent controls

Mortality rate nil " " " " 30.0 " " "

Each fowl bitten by two mosquitoes 19 to 25 days after their infective meal

N = negative smear, D = died of malaria, SR = spontaneous recovery, + = positive smear but less than 1 per cent of red cells infected. Counted as 1 per cent in totals

In Table IX are shown data from the second series (346, 353, 357, 388, 389, 393, 401-3, 409) having both serum and vaccination treatment. Each fowl, between June 19 and July 18, received intravenous injections of saline suspensions of salivary glands dissected from 40 infective mosquitoes and exposed

to ultraviolet radiation for 30 minutes to inactivate the sporozoites Fowl 346 received glands from a total of 280 infective mosquitoes, fowls 353, 357, 388 from 240, and the others from 200 infective mosquitoes, respectively

TABLE IX

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Serum and Also of Sporozoite Vaccine Second Series of Combined Treatments

Fowl No.	Weight of fowl gms	Agglutinating titre of fowl serum	Prepatent period days	Percentage of red blood cells infected																															Result
				Days following infection																															
				7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34				
Experimental																																			
346	1005	262 144	8	N	N	+	+	1	3	3	8	5	2	+	+	N	N															SR			
353	635	262 144	12	N	N	N	N	N	N	+	2	3	6	9	4	+	+	N	N													SR			
357	747	262 144	9	N	N	N	+	1	4	5	8	3	+	+	N	N																SR			
358	—	131 072	11	N	N	N	N	N	+	4	28	53	60	51	63	D																D			
389	—	131 072	8	N	N	+	+	+	4	9	7	4	+	N	N																	SR			
393	692	131 072	12	N	N	N	N	N	N	+	2	3	23	35	8	+	+	+	+	+	+	+	+	+	N	N						SR			
401	914	131 072	12	N	N	N	N	N	N	+	+	4	7	7	3	2	+	+	+	+	+	N	N									SR			
402	843	131 072	20	N	N	N	N	N	N	N	N	N	N	N	N	N	+	+	2	9	13	8	2	N	N							SR			
403	—	131 072	10	N	N	N	N	+	+	3	9	9	2	+	N	N																SR			
409	—	131 072	11	N	N	N	N	N	+	2	8	21	41	D																		D			
Control																																			
413	629	—	9	N	N	N	+	+	+	1	3	2	+	+	+	N	N															SR			
414	657	—	10	N	N	N	N	+	+	1	5	6	3	+	N	N																SR			
415	612	—	20	N	N	N	N	N	N	N	N	N	N	N	N	N	N	+	+	1	8	28	24	25	20	19	6	+	+	N	N	SR			
416	538	—	9	N	N	N	+	1	2	7	17	6	+	N	N																	SR			
417	—	—	9	N	N	N	+	2	8	5	2	+	N	N																		SR			
418	597	—	8	N	N	+	+	3	6	5	2	+	N	N																		SR			
419	461	—	8	N	N	+	+	8	28	30	16	+	+	N	N																	SR			
420	702	—	10	N	N	N	N	+	2	6	15	+	+	N	N																	SR			
421	453	—	8	N	N	+	+	2	6	1	+	+	N	N																		SR			
422	—	—	8	N	N	+	+	5	14	23	41	60	72	73	41	29	19	23	33	65	72	83	71	57	50	D						D			
423	736	—	9	N	N	N	+	+	5	4	+	+	+	3	2	+	N															SR			
424	—	—	9	N	N	N	+	+	3	31	35	15	1	N	N																	SR			

Average prepatent period 11.3 days experimental fowls, 9.8 days control

" highest infections 20.4 per cent experimental fowls, 20.2 per cent controls.

Mortality rate 20.0 " " " 8.3 " "

Each fowl bitten by two mosquitoes 20 to 25 days after their infective meal.

N = negative smear D = died of malaria SR = spontaneous recovery + = smear positive but less than 1 per cent of red cells infected Counted as 1 per cent in totals.

From June 14 to 18 each fowl received an injection of 1 cc. of fowl serum pooled from fowls having a chronic infection with the homologous *Plasmodium*. The injections were given intravenously and intraperitoneally, alternately. Tests of the agglutinating titre of these fowls against homologous sporozoites were made on July 20. The results are shown in Table IX. In no case was

the titre less than 1/131,072 and in three cases it was at 262,144, which is a high dilution

Each fowl, experimental and control, was bitten on July 20 by two infective *Aedes albopictus* mosquitoes 20 to 25 days after the insects had had their infective blood meal. As shown in Table IX the average prepatent period in this

TABLE X

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Serum and Also of Sporozoite Vaccine Third Series of Combined Treatments

Fowl No	Weight of fowl	Prepatent period	Percentage of red blood cells infected																Result
			Days following infection																
			7	8	9	10	11	12	13	14	15	16	17	18	19	20			
	gm	days																	
<i>Experimental</i>																			
A 46	825	10	N	N	N	N	+	+	1	6	8	3	+	N	N			SR	
A 48	809	8	N	N	+	+	2	5	5	+	+	N	N					SR	
A 50	722	9	N	N	N	+	+	2	15	29	55	D						D	
A 51	742	8	N	N	+	1	8	6	1	+	+	N	N					SR	
A 53	888	7	N	+	+	+	1	5	2	+	+	N	N					SR	
A 60	874	7	N	+	+	+	5	10	12	1	+	+	N	N				SR	
<i>Control</i>																			
A 93	868	7	N	+	+	5	7	23	44	32	D							D	
A 94	900	7	N	+	+	+	6	12	13	8	1	+	+	N	N			SR	
A 95	810	9	N	N	N	+	3	11	21	27	51	D						D	
A 96	799	8	N	N	+	+	+	6	17	33	D							D	
A 97	795	8	N	N	+	+	2	7	30	41	19	11	2	+	N	N		SR	
A 98	833	8	N	N	+	1	17	12	5	1	+	+	N	N				SR	

Average prepatent period 8.2 days experimental fowls, 7.8 days controls

" highest infections 15.5 per cent experimental fowls, 33.2 per cent controls

Mortality rate 16.7 " " " " 50.0 " " "

Each fowl bitten by two mosquitoes 15 days after their infective meal

N = negative smear, D = died of malaria, S R = spontaneous recovery, + = smear positive but less than 1 per cent of red cells infected. Counted as 1 per cent in totals

second series was 11.3 days, the mortality rate was 20.0 per cent, and the average of highest percentages of red cells infected was 20.4 per cent

In Table X are shown the data for the third series (A 46, 48, 50, 51, 53, 60) having combined prophylactic treatment. Each fowl received five intravenous injections of a saline suspension of ground dried thoraces of infective mosquitoes, between August 20 and September 13. A total of 200 mosquitoes was used for each fowl. From September 15 to 20 each fowl received daily 1 cc intraperitoneally of pooled sera from fowls chronically infected with the

homologous *P. gallinaceum*. On September 17 each fowl was bitten by two infective *A. albopictus* 15 days after the insects had had their infective blood meal. Thus there were three injections of serum after the mosquitoes had

TABLE XI

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Serum and Also of Sporozoite Vaccine Fourth Series of Combined Treatments

Fowl No	Weight of fowl	Prepatent period	Percentage of red blood cells infected																							Result
			Days following infection																							
			8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25						
	gm	days																								
Experimental																										
B 5	825	12	N	N	N	N	N	+	+	+	5	9	5	+	+	N	N									SR
B 6	765	8	N	+	+	+	4	18	20	24	11	4	4	+	+	N	N									SR
B 7	918	11	N	N	N	N	+	+	2	+	+	N	N													SR
B 9	764	10	N	N	N	+	+	2	4	8	3	+	N	N												SR
B 10	974	10	N	N	N	+	+	7	4	+	N	N														SR
B 12	747	10	N	N	N	+	+	2	8	9	4	+	N	N												SR
B 13	879	10	N	N	N	+	+	2	5	4	+	N	N													SR
B 16	811	11	N	N	N	N	+	+	4	10	13	2	N	N												SR
B 19	902	11	N	N	N	N	+	+	9	8	+	N	N													SR
B 21	1 192	11	N	N	N	N	+	+	+	+	N	N														SR
B 22	994	10	N	N	N	+	+	5	8	6	+	+	N	N												SR
B 23	832	8	N	+	+	2	3	2	+	+	+	+	N	N												SR
B 26	763	9	N	N	+	+	8	23	29	15	1	+	N	N												SR
B 31	797	10	N	N	N	+	+	2	4	5	+	N	N													SR
B 32	742	11	N	N	N	N	+	+	4	1	+	N	N													SR
Control																										
B 76	779	9	N	N	+	+	4	27	31	D																D
B 77	939	10	N	N	N	+	+	1	2	8	22	D														D
B 78	784	9	N	N	+	+	6	21	25	D																D
B 79	890	9	N	N	+	+	3	5	9	18	23	32	39	D												D
B 80	811	8	N	+	2	6	21	35	28	21	18	12	16	43	54	62	67	63	57	D						D
B 81	608	9	N	N	+	+	4	15	38	61	66	D														D

Average prepatent period 10.1 days experimental fowls, 9.0 days controls.

highest infections 9.0 per cent experimental fowls, 41.7 per cent controls.

Mortality rate nil 100.0

Each fowl bitten by two mosquitoes eleven days after their infective meal.

N = negative smear D = died of malaria SR = spontaneous recovery + = smear positive but less than 1 per cent. Counted as 1 per cent in totals.

infected the fowls. There were six controls (A 93-98) each bitten by two infective mosquitoes in the same lot.

As shown in Table X, the average incubation periods were 8.2 and 7.8 days

in experimental and control fowls, respectively. The mortality rate in the former was 16.7 per cent and in the latter 50.0 per cent. The average of highest percentages of red cells infected was 15.5 in the experimental fowls and 33.2 in the controls.

The fourth series (B 5-7, 9, 10, 12, 13, 16, 19, 21-23, 26, 31, 32) having combined prophylactic treatment is shown in Table XI. Each of these 15 fowls received six intravenous injections of saline suspensions of ground dried thoraces from infective mosquitoes between September 19 and October 16, a total of 240 mosquitoes being used for each fowl. Daily intraperitoneal

TABLE XII

Mosquito-Borne Infections of P. gallinaceum in Fowls Which Had Previously Received Injections of Serum and Also of Sporozoite Vaccine. Four Series Combined

Series No	Serial Nos. of fowls	No. of fowls in series	Deaths from malaria	Spontaneous recoveries	Mortality rate	Prepatent period		Highest percentages of red cells infected		Remarks
						Total	Average for series	Total	Average for series	
						days	days			
1	231, 233-239, 241, 272	10	0	10	Nil	82	8.2	183	18.3	See Table VIII
2	346, 353, 357, 388, 389, 393, 401-3, 409	10	2	8	20.0	113	11.3	204	20.4	See Table IX
3	A 46, 48, 50, 51, 53, 60	6	1	5	16.7	49	8.2	93	15.5	See Table X
4	B 5-7, 9, 10, 12, 13, 16, 19, 21-23, 26, 31, 32	15	0	15	Nil	152	10.1	135	9.0	See Table XI
Totals.		41	3	38	7.3	396	9.7	615	15.0	

injections of 1 cc. of pooled sera from fowls chronically infected with the homologous *Plasmodium* were given to each fowl from October 20 to 26. On October 22 each fowl was bitten by two infective *A. albopictus* mosquitoes, 11 days after these insects had had their infective blood meal. Thus there were four injections of serum after the fowls were infected by the mosquitoes. There were six controls (B 76-81) each of which was bitten by two infective mosquitoes of the same lot.

As shown in Table XI the average prepatent periods were 10.1 days in the experimental fowls and 9.0 in the controls. The mortality rate in the former was nil while in the latter it was 100 per cent. The average of highest per-

centages of infected red cells in the experimental fowls was 9.0 and in the controls 41.7

In Table XII the four series that had combined treatment have been grouped together. The total of 41 fowls had an average prepatent period of 9.7 days, an average mortality of only 7.3 per cent, and an average highest percentage of infected red cells of 15.0 per cent.

DISCUSSION

The data for the grouped series of experiments can be briefly summarized as follows—

Description	Total No. of fowls	Average prepatent period	Average mortality rate	Average highest percentage of red cells infected (daily counts)	Average percentage red cells infected just prior to death
		days	per cent		
Normal fowls (Table III)	83	9.1	55.4	30.1*	44.6
Fowls having sporozoite vaccination (Table II)	19	8.9	21.1	20.5†	54.7
Fowls having serum injections (Table VII)	18	8.9	16.7	17.9	45.0
Fowls having both sporozoite vaccination and serum injections (Table XII)	41	9.7	7.3	15.0	53.7

* Based on observations on 55 fowls.

† Based on observations on 10 fowls.

Several interesting points emerge. In the first place, none of the experimental procedures in these attempts to immunize fowls against *P. gallinaceum* had any great effect on the duration of the prepatent period which has varied between 7 and 20 days. There was some evidence of a slight prolongation of this period in the series receiving combined treatment and reported in Tables IX and XI. Here the prepatent periods averaged 11.3 and 10.1 days, respectively.

The prepatent period following mosquito-borne infection was longer than that following intravenous inoculation by trophozoites as has been noted by others. In the latter infections the prepatent period varied from 1 to 6 days with an average of 2.8 days in 12 fowls. The larger the inoculation of trophozoites the shorter the period.

In the second place, this report confirms the earlier results in indicating that fowls can be actively immunized to some extent against mosquito-borne infections of *P. gallinaceum* by vaccination with homologous inactivated sporozoites. In the first report the mortality rate in six vaccinated fowls with sporozoite agglutinating titres of 1/32,768 or higher was nil (Mulligan *et al.*, 1941).

But only two of these (127 and 150) had been fed on by two mosquitoes. The others were bitten by only one infective mosquito. Seven more cases were reported in the second report (Russell *et al.*, 1942) all bitten by two mosquitoes but using mosquitoes probably, but not certainly, 20 days after their infective blood meal. The mortality rate was 14.3 per cent in this series. In the third series reported here for the first time (Table I) there were 10 fowls, fed on by two mosquitoes 15 days after infective meal, and the mortality rate was 30 per cent. The combined average mortality rate for the 19 fowls was 21.1 per cent, which was less than half that of normal fowls similarly infected. This is a significant measure of immunization, although in no case was infection prevented.

Thirdly, we have shown (Table VII) that serum injections produced a similar partial immunity, so that an average mortality of 16.7 per cent prevailed. As will be reported elsewhere (Russell and Mohan, 1942) we know that such serum injections, whether of normal sheep serum or of sera pooled from fowls infected with the homologous *Plasmodium*, will enlarge the fowl spleen to nearly double its normal volume. On section these enlarged spleens showed a histological picture which, except for absence of parasites, pigment, thrombi, and infarcts, was analogous to that of the enlarged spleen of acute malaria (See also Manwell and Goldstein, 1940). Therefore, we believe that by the serum injections we stimulated the cellular defence mechanism against malaria. Whether or not the humoral elements were also stimulated, and to what extent there was passive immunity, we cannot say. But in one series (Table IV) there was no evidence of any rise in the agglutinating titre of the fowl serum against homologous sporozoites.

Fourthly, it was in the grouped series of 41 fowls (Table XII) which had had both humoral stimulation by sporozoite vaccination and cellular stimulation by serum treatment that we see the greatest immunizing effect. In these fowls the average mortality was only 7.3 per cent, as contrasted with a normal mortality of 55.4 per cent. The numbers in each group were large enough so that this difference in immunity rates is statistically highly significant. During the course of infection in these birds the percentage of red cells found infected in daily counts was never over 50 per cent except in two of the three fatalities (388 and A 50) in which it was 63 and 55 per cent, respectively. The average of the highest daily percentages of red cells infected in this group of 41 fowls was 15.0 per cent as contrasted with 30.1 per cent for normal fowls. The average percentage of red cells infected immediately prior to death was a little higher in the vaccinated groups than in the controls.

The course of infection in the three series of experimental fowls and in the controls is presented in Table XIII, where the average percentages of red cells infected are shown by days throughout infection, in the fowls which spontaneously recovered. The data have been plotted in Chart 1. It will be noted

Course of Infection of *P. gallinaceum* in Fowls, As Marked by Average of Daily Percentages of Red Blood Cells Infected. Non Fatal Cases

Serial Nos. of fowls	No of fowls	Percentages of red blood cells infected																															
		Days following infection																															
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33					
<i>Controls</i>																																	
370, 1, 3 5-8	7	0	0	3	5	21	45	56	44	17	7	3	3	2	0	0		1	1	0	0												
395 8, 9 400	4	0	0	1	3	3	15	32	17	11	11	23	40	30	25	7	1	1	0	0													
413-21 23 24	11	0	0	2	8	21	62	91	97	35	8	5	3	1	0	2	2	5	9	29	24	25	20	19	6	1	1	0					
A 60-41	2	0	0	0	1	2	6	2	1	2	4	3	2	1	0	1	0	0															
A 94 97-98	3	0	1	3	3	25	31	48	50	21	13	3	1	0	0																		
D 2, 5 6	3	0	0	2	3	12	24	32	12	6	2	1	0	0																			
Totals	30	0	1	12	23	81	183	261	221	92	45	38	49	34	25	10	3	6	10	29	21	25	20	19	6	1	1	0					
Average		0	—	0.4	0.8	2.8	6.1	8.7	7.4	3.1	1.5	1.3	1.6	1.1	0.8	0.3	0.1	0.2	0.5	1.0	0.8	0.8	0.7	0.6	0.2	—	—	0					
<i>Serum-treated fowls with spontaneous recovery</i>																																	
359 60 62, 68	4	0	0	1	2	3	9	25	40	47	53	18	15	6	4	2	1	1	1	3	9	13	15	28	19	10	3	1	0				
A 91 B 64-68	6	0	0	5	6	12	30	25	32	32	14	8	2	1	1	1	1	0	0														
B 85 91 93 96, 100	5	0	0	2	10	21	38	60	43	30	26	5	2	0	0																		
Totals	15	0	0	8	18	36	77	110	115	109	73	31	19	7	5	2	2	1	3	9	13	15	28	19	10	3	1	0					
Average		0	0	0.5	1.2	2.4	5.1	7.3	7.7	7.3	4.9	2.1	1.3	0.5	0.3	0.1	0.1	0.1	0.2	0.6	0.9	1.0	1.9	1.3	0.7	0.2	0.1	0					
<i>Vaccinated fowls with spontaneous recovery</i>																																	
A 2 4, 7 11 15 22	7	0	0	3	4	19	29	31	24	12	6	1	1	1	1	1	0	0															
Average		0	0	0.4	0.6	2.7	4.1	4.4	3.4	1.7	0.9	0.1	0.1	0.1	0.1	0.1	0																
<i>Vaccinated and serum-treated fowls with spontaneous recovery</i>																																	
231 3-9 41 77	10	0	2	16	37	85	109	101	71	51	31	17	11	5	5	2	1	1	1	0	0												
346, 353 357 389 393 401 402, 403	8	0	0	2	3	4	12	23	37	33	42	54	16	4	3	3	3	4	10	14	9	3	0	0									
A 40 48, 51 53 60	5	0	2	4	4	17	27	21	10	12	4	1	0	0																			
D 5-7 9 10 12 13 16 19 21 23 26, 31 32	13	0	0	2	3	10	26	69	104	95	44	19	9	2	2	0	0																
Totals	48	0	4	24	47	116	14	214	222	191	121	91	36	11	10	5	4	5	11	14	9	3	0	0									
Average		0	0.1	0.6	1.2	3.1	4.6	5.6	5.8	5.0	3.2	2.4	0.9	0.3	0.3	0.1	0.1	0.1	0.3	0.4	0.2	0.1	0	0									

that the partially protected fowls did not show as high a peak of infection as the controls and in the case of the fowls having serum and in those having both serum and vaccination the peak came on the 14th day and not on the 13th as with the controls

The crisis, so marked in the controls, with a very rapid fall on the 14th to 17th days was not so rapid in the protected birds. For instance, in the group

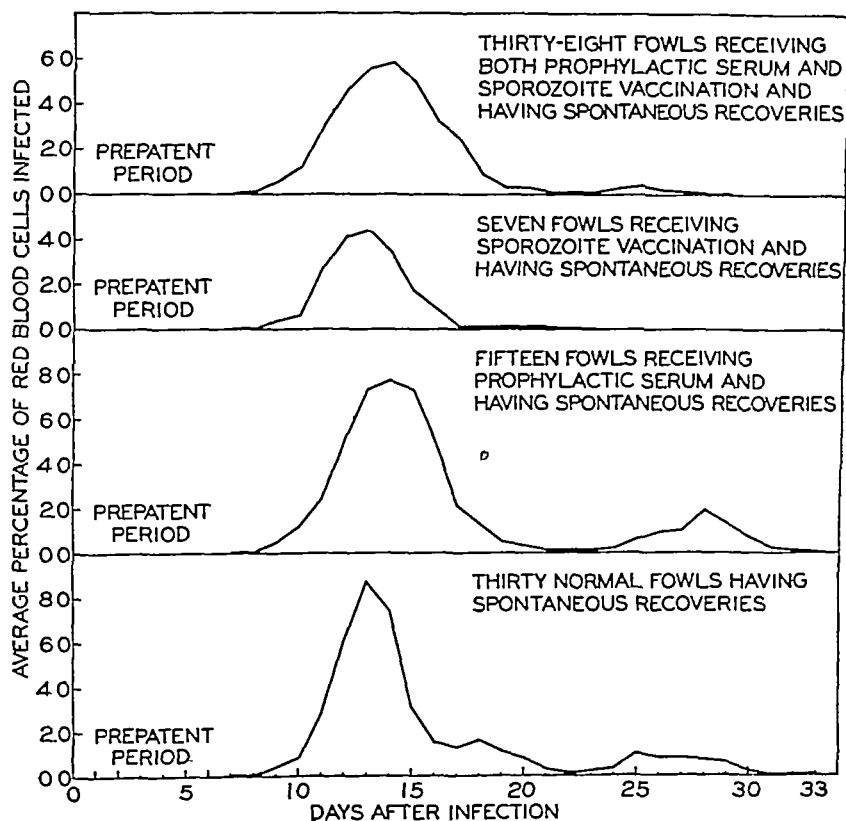


CHART 1 Course of infection in fowls with mosquito-borne *Plasmodium gallinaceum*, normal and with prophylactic treatment

having both serum and vaccination the corresponding fall extended from the 15th to 19th day

While we did not succeed in preventing infection in any fowl by the combined prophylactic treatment our test was a severe one, for the infecting dose of two mosquitoes was a relatively large one. We believe the results tend to confirm and extend in fowls the findings of Mulligan *et al* (1940) in monkeys, which demonstrated the interaction of both cellular and humoral agencies in defence against malaria

SUMMARY AND CONCLUSIONS

This paper reports attempts to immunize domestic fowls against mosquito borne infections of *Plasmodium gallinaceum* by means of (a) vaccination with inactivated homologous sporozoites, (b) injections of sera, both normal sheep serum, and serum from fowls chronically infected with the homologous *Plasmodium*, (c) combinations of both sporozoite vaccine and serum

It was possible to reduce the normal malaria death rate (55.4 per cent) in these fowls by each of the above methods but most markedly by the combined prophylactic treatment. Mortality rates were 21.1 per cent in vaccinated fowls, 16.7 per cent in serum treated fowls, and 7.3 in those having the combined treatment

Intensity of infection was measured by counting the percentage of red cells infected each day. It was found that in each group of fowls having prophylactic treatment the average of highest percentages of red cells infected was less than in untreated malarious fowls (30.1 per cent). The average figure was 20.5 per cent in vaccinated fowls, 17.9 per cent in those having serum injections, and 15.0 per cent in those having combined treatment

The prepatent period was not markedly affected by any of the prophylactic procedures. It averaged 9.1 days in the untreated group, 8.9 days in both the vaccinated and serum treated groups, and 9.7 days in the group having combined treatment

The results seemed to demonstrate an interaction of both cellular and humoral agencies in defence against malaria, since the greatest immunizing effect was seen in the series having both sporozoite vaccine and serum injections

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THE SPECIFIC POLYSACCHARIDE CONTENT OF PNEUMONIC SPUTA*

By J T TRIPP Ph.D A. W FRISCH, M.D, C D BARRETT, JR., AND
B E. PIDGEON

(From the Michigan Department of Health Laboratories, Lansing, and the Department of Bacteriology and Clinical Pathology College of Medicine, Wayne University Detroit)

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The microscopic examination of sputum from patients with pneumonia has revealed that Type III infections are distinctly different from those due to other pneumococci. The evidence obtained supported the hypothesis that the prognosis was primarily dependent upon the amount of Type III capsular carbohydrate produced and secondarily upon the number of organisms present in the pneumonic exudate (1). The character of the visualized background in Wright stained smears of rusty sputum was the first means of classifying patients with Type III pneumonia into two major groups. In one group called "non reticulated" the usual granular pink background was present on the slides as contrasted with a second group called "reticulated" in which the background was composed of a branching, fibrin like network which appeared to originate from the similarly stained capsules of the interdispersed pneumococci. In the non reticulated instances the bacteremic incidence was 7 per cent and the fatality rate was 9 per cent, whereas in the reticulated group the bacteremic incidence was 65 per cent and the fatality rate was 83 per cent (2). It was then found that by the addition of as little as 0.02 mg. of Type III S to 0.1 cc. of any pneumonic sputum an artificial reticulation could be produced which closely resembled that seen in the naturally occurring pneumonia. Further evidence that the reticulation was due to an unusually large amount of carbohydrate was obtained by means of the "quellung" reaction. Neufeld preparations of reticulated specimens of sputum revealed a meshwork of excess capsular material either in the form of swollen free masses, or in the form of strands connecting one pneumococcus to another (2). The striking differences in the amount of S observed in the "quellung" reaction suggested that quantitative methods could be utilized. Therefore, in further test of the above hypothesis, specimens of sputum from patients with Type I, II, III, VII, and VIII pneumonia were analyzed for their capsular polysaccharide content by a method based on the work of Avery and Goebel (3), Heidelberger, Kendall, and Scherp (4), and Wadsworth and Brown (5).

* Supported by a grant from the Commonwealth Fund to the Michigan Department of Health Laboratories

Methods

Samples of sputum were obtained prior to and within 48 hours from the beginning of therapy. To insure that the samples originated from the pneumonic areas in the lung, only rusty or bloody specimens were analyzed. Each specimen was made homogeneous with the aid of glass beads and a mechanical shaker. To an accurately measured aliquot (10 ml if available) was added an equal volume of acetate buffer solution,¹ glass beads, and a drop of caprylic alcohol to prevent foaming. This mixture was then shaken vigorously for 30 minutes. Two volumes of 95 per cent alcohol were added slowly and the sample placed in the cold room overnight. The next morning the samples were centrifuged, the supernate discarded, and the residue was extracted with 10 ml of acetate buffer in the manner described above. The mixture was centrifuged, the supernate decanted, and the insoluble fraction was extracted with acetate buffer until the washings no longer gave a positive ring test with homologous antiserum. The acetate buffer washings were then combined, precipitated with two volumes of 95 per cent alcohol, and the centrifuged precipitate reextracted and reprecipitated twice more for purification. The last alcohol precipitate was dissolved in slightly acidified, warm saline which was subsequently neutralized to litmus with dilute NaOH. The purified solution of S was then adjusted to the original volume of the sputum aliquot.

A modification of the Heidelberger and Kendall procedure was employed to measure the amount of S in the sputum extracts (6, 7). A given antiserum for each type being studied was standardized with known amounts of a highly purified polysaccharide. The amount of nitrogen precipitated was plotted against the corresponding amounts of S. The analysis of an unknown solution of S must be conducted in the region of antibody excess (7). The quantity of S in the unknown purified sputum aliquots was adjusted to fall within this range. This was accomplished by comparing the relative precipitin titers of the unknown with a standard solution of S. In general the samples with the highest titers contained the most carbohydrate, but the well known limitations of the precipitin test prohibited its use for detecting small differences. Triplicate 1 ml aliquots of the unknown S solutions were added to 1 ml quantities of standard antiserum and the precipitable nitrogen determined by the micro Kjeldahl technique. On the basis of these values the quantity of S in the sample was read from the standard curve.

The activities of the S preparations used in standardizing the serums were as follows —

		Precipitin titer	Antibody N precipitated by 0.1 mg S	Potency of homologous antiserum
			mg/ml	mg N/ml
S I	Lot 2	1 10,000,000	1.0	2.0*
S II	Lot 3	1 5,000,000	1.2	2.1*
S III	Lot 5	1 5,000,000	1.1	2.1†
S VII	Lot 3	1 2,000,000	0.7	2.3*
S VIII	Lot 2	1 5,000,000	1.4	2.1*

* Horse serum

† Rabbit serum

¹ Sodium chloride 0.9 per cent, sodium acetate 4 per cent, glacial acetic acid 2 per

The final results of the sputum analyses are reported in terms of mg per cent. The practice of calculating the results to the basis of dry weight of the sample was abandoned because in 17 such analyses there were no significant differences between the two sets of data.

RESULTS

In Table I are presented the results of the analyses of 19 samples of sputum from 14 patients with Type I pneumonia. It can be noted that the cases are relatively mild as judged from the degree of involvement, incidence of bacteremia, and leukopenia. The sputum counts represent the highest number

TABLE I
Specific Polysaccharide Content of Type I Sputa

Patient No	Age	Bacteremia	Leukopenia	Multiple lobe	Sputum count†	S	Outcome
	yr					mg per cent	
1	39	—	+	+	13	0.8	L
2	24	—	—	—	1	0.8	L
3	30	+	—	—	14	1.1	L
4	38	—	—	+	15	1.2	L
5	35	—	—	—	12	1.2	L
6	60	—	—	—	5	1.3	L
7	48	—	—	—	26	1.3	L
8	39	+	—	+	57	1.3	L
9	16	+	—	—	3	1.8	L
10	40	—	+	—	25	1.8	L
11	32	—	—	+	8	2.0	L
12	60	—	—	—	14	2.5	L
13	67	+	—	+	55	2.6	D
14	29	—	—	—	23	5.3	L

* The term leukopenia refers to those patients with total leukocyte counts of less than 10 000 during the acute stages of the pneumonia.

† Refers to the highest number of pneumococci per oil immersion field during the period when sputum specimens were collected.

of pneumococci per oil immersion field in Wright stained smears from the same specimens which were analyzed. The single death in the series was due to cardiac failure. The amount of Type I pneumococcus polysaccharide in the sputum varied from 0.8 to 5.3 mg per cent with an average of 1.8. The maximum concentration of Type I S encountered was 10.4 mg per cent in lung exudate obtained at autopsy.² Sufficient sputum could not be obtained from this patient to make an analysis.

Table II presents the results of the analyses of 20 specimens of sputum from 10 patients with Type II pneumonia. It can be noted that the cases were more severe in character as judged from the clinical and sputum data. The amounts of specific carbohydrate varied from 1.0 to 94 mg per cent with

² The results of the autopsy analyses will be reported in a separate communication.

an average of 16.1. The sputum from patient 24 contained numerous pneumococci and the value of 94 mg. was only exceeded by a value of 112 mg. per cent in the lung exudate from the same patient at autopsy.

TABLE II
Specific Polysaccharide Content of Type II Sputa

Patient No.	Age	Bacteremia	Leukopenia	Multiple lobe	Sputum count	S	Outcome
	yrs					mg. per cent	
15	43	—	—	—	5	1.0	L
16	56	+	+	+	12	1.1	L
17	43	—	+	+	48	3.9	L
18	61	+	+	+	23	8.3	D
19	30	+	—	+	48	8.6	L
20	66	+	+	+	18	8.6	L
21	21	+	—	+	40	9.8	L
22	35	+	—	+	16	12.2	L
23	59	+	+	+	100±	13.2	D
24	33	+	+	+	200±	94.0	D

TABLE III
Specific Polysaccharide Content of Type VII Sputa

Patient No.	Age	Bacteremia	Leukopenia	Multiple lobe	Sputum count	S	Outcome
	yrs					mg. per cent	
25	38	+	—	+	10	1.9	L
26	25	—	—	—	8	2.3	L
27	26	+	—	+	14	3.0	L
28	39	—	—	+	4	4.2	L
29	23	+	+	+	30	5.5	L
30	64	—	—	—	15	7.0	L
31	38	+	—	+	60	7.0	L
32	23	+	—	+	5	9.0	L
33	20	—	—	—	3	10.5	L
34	46	—	—	+	4	18.5	L
35	44	+	+	+	200±	20.0	D
36	56	+	+	+	200±	76.0	D

Table III presents the analytical results of 16 specimens from 12 patients with Type VII pneumonia. The amounts of specific polysaccharide varied from 1.9 to 76 mg. per cent with an average of 13.7. The greatest amounts of Type VII S were obtained from the sputa of cases 35 and 36 and the maximum value of 76 mg. was only exceeded by a concentration of 96 mg. per cent in the lung exudate of patient 36 at autopsy.

Table IV presents the analyses of 16 specimens of sputum from 11 patients

TABLE IV
Specific Polysaccharide Content of Type VIII Sputa

Patient No	Age	Bacteremia	Leukopenia	Multiple lobe	Sputum count	S	Outcome
	yr					mg per cent	
37	39	+	-	+	23	0.5	L
38	29	-	+	+	12	1.0	L
39	36	-	-	-	3	1.0	L
40	38	-	-	-	25	1.4	L
41	42	+	+	+	18	1.7	L
42	40	-	-	-	6	2.1	L
43	33	-	+	-	13	3.2	L
44	43	+	+	-	26	3.8	L
45	66	+	-	+	22	3.9	D
46	53	-	+	+	15	6.2	L
47	58	-	-	-	3	6.8	L

TABLE V
Specific Polysaccharide Content of Non Reticulated Type III Sputa

Patient No	Age	Bacteremia	Leukopenia	Multiple lobe	Sputum count	Excess S*	Reticulation†	S	Outcome
	yr							mg per cent	
48	52	-	-	-	5	-	-	4.0	L
49	37	-	+	+	15	+	-	4.8	L
50	33	-	-	-	<1	-	-	6.0	L
51	39	-	-	-	7	-	-	6.0	D
52	30	-	-	+	13	-	-	8.0	L
53	55	-	-	-	9	-	-	8.4	L
54	57	-	-	-	<1	-	-	14.0	L
55	46	-	-	-	<1	-	-	20.0	L
56	48	-	-	-	4	-	-	24.0	L
57	23	-	-	-	<1	-	-	25.0	L
58	38	-	-	-	4	-	-	26.0	L
59	36	-	-	-	1	-	-	26.0	L
60	40	-	-	-	13	-	-	30.0	L
61	10	-	-	-	17	-	-	32.0	L
62	43	-	-	-	<1	+	-	44.0	L
63	57	-	-	-	24	+	-	48.0	L
64	38	-	-	-	7	-	-	68.0	L
65	23	-	-	-	5	+	-	92.0	L
66	42	-	-	-	6	-	-	92.0	L
67	49	-	-	+	51	+	-	300.0	L

* Excess S refers to the amount of capsular carbohydrate observed during typing.

† Classification on the basis of Wright stained sputum smears (1-2)

- signifies that all S present is in the capsules of the pneumococci.

+ or ++ signifies that S present is in capsules and in strands connecting two or more pneumococci.

+++ or ++++ signifies that large amounts of S in the form of strands and free masses are present.

with Type VIII pneumonia. The patients in this group were only mildly ill as judged from the clinical data and sputum counts. The single death was attributable to pericarditis and empyema. The amounts of specific polysaccharide varied from 0.5 to 6.8 mg per cent with an average of 2.9. The maximum value of Type VIII S was 25.2 mg per cent obtained at autopsy from a patient whose sputum was not analyzed.

Table V lists the results of 32 specimens of sputum from 20 patients with Type III pneumonia. These cases were classified as non-reticulated on the

TABLE VI
Specific Polysaccharide Content of Reticulated Type III Sputa

Patient No	Age	Bacteremia	Leukopenia	Multiple lobe	Sputum count	Excess S	Reticulation	S <i>mg per cent</i>	Outcome
68	61	—	—	—	29	++	+	18.0	L
69	54	+	—	+	16	++++	++++	28.0	D
70	33	+	+	+	12	+	+	72.0	L
71	52	—	+	+	59	+	+	130.0	D
72	63	+	+	+	74	++	+	180.0	L
73	53	—	—	+	36	++	+	240.0	L
74	72	—	+	+	38	++	+	244.0	D
75	67	—	+	+	19	++	++	300.0	D
76	—	+	—	+	4	++	++	340.0	D
77	67	+	+	+	53	++++	++++	400.0	D
78	54	—	—	—	82	+++	+++	640.0	L
79	64	—	+	—	13	++	++	640.0	D
80	48	—	—	—	16	++++	++++	800.0	D
81	42	+	—	+	29	++++	++++	1,200.0	D
82	52	+	—	+	36	++++	++++	1,440.0	D
83	49	+	+	—	100±	++++	++++	2,120.0	D
84	49	—	+	+	73	++++	++++	2,200.0	D
85	68	+	—	+	44	++++	++++	4,800.0	D
86	50	+	+	+	25	++++	++++	10,000.0	D

basis of Wright's stained smears of sputum but they were further subdivided depending upon the amount of excess polysaccharide observed during the Neufeld typing. The clinical data and outcome emphasize the mild character of the disease but the amounts of Type III S recovered from the sputa varied from 4 to 300 mg per cent with an average of 45.

The above results are to be compared with those in Table VI which represent the analyses of 29 specimens of sputum from 19 patients who were classified as reticulated and in whom the typing reaction revealed an excess of polysaccharide. This group contained the majority of severely ill Type III patients as can be seen from the incidence of bacteremia, leukopenia, multiple lobe involvement, and fatality rate. The amounts of specific polysaccharide varied

from 18 to 10,000 mg per cent with an average of 1,360. The maximum value of 10,000 mg per cent was the largest amount of Type III S obtained from all of the specimens examined.

DISCUSSION

To the authors' knowledge the above results represent the first attempt to determine quantitatively the amount of capsular polysaccharide in rusty or bloody sputum. We do not wish to imply that the values obtained are absolute. In addition to errors in sampling, extraction, and those inherent in the method, the final figures could also vary depending upon the activity of the purified S used in the preparation of the standard curves. In general it can be said that the S values obtained both from the reticulated and non reticulated Type III sputums were much higher than had been anticipated. The average for the reticulated Type III cases roughly exceeded by 170 times the amount of S recoverable from the sputa of cases of Type I, II, VII, and VIII pneumonia. The values in the non reticulated cases were approximately 5 times those obtained for the other types. The data again emphasize the atypical character of Type III pneumonia.

It can be noted from Tables V and VI that the Neufeld typing reaction is a more delicate indicator of the presence of excess S in the sputum than is the Wright stain. The latter, however, is a more dependable prognostic aid. Strands of polysaccharide connecting pneumococci began to be evident in the sputum when the concentrations of S approached 40 mg per cent whereas, between 100 and 200 mg of S were usually required before reticulation became apparent. A few exceptions to this general rule may have been due to errors in extraction or to inadequate specimens. The number of pneumococci per oil immersion field (sputum count) was generally higher in the reticulated than in the non-reticulated cases and coincided with the amount of S recoverable from the sputum. This finding, however, did not appear to hold for infections due to Types I, II, VII, and VIII pneumococci. No obvious explanation for this discrepancy is available at present. The data on sputum counts and analyses in Type III pneumonia suggest that the amount of S elaborated, together with the number of pneumococci in the sputum, should be considered in order to evaluate individual cases. The relationship between the sputum and autopsy analyses together with additional discussion of the total results will be reported in a further communication.

CONCLUSIONS

- 1 The average specific polysaccharide content of rusty or bloody sputa in Type III was 91 times greater than the average for Types I, II, VII, and VIII pneumonia.

- 2 Those Type III sputums which were classified as reticulated contained

an average S concentration of 1,360 mg per cent or 170 times more than the amount found in other types

3 Those Type III sputa which were classified as non-reticulated contained an average S concentration of 45 mg per cent or 5.5 times more than the amount found in other types

4 The amount of specific polysaccharide in the sputa of patients with Type III pneumonia furnishes an index to the severity of the disease and an aid in prognosis

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THE SPECIFIC POLYSACCHARIDE CONTENT OF PNEUMONIC LUNGS*

By A. W. FRISCH, M.D., J. T. TRIPP Ph.D., C. D. BARRETT, JR., AND B. E. PIDGEON
(From the Departments of Bacteriology and Clinical Pathology, College of Medicine,
Wayne University, Detroit and the Michigan Department of Health Laboratories
Lansing)

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The atypical behavior of Type III pneumonia can be explained by the hypothesis that the particular pneumococcus responsible for the disease frequently produces relatively large amounts of capsular carbohydrate as the organism grows in the lung. The evidence to support this concept was presented in a previous communication in which it was reported that the S content of Type III sputa exceeded by 91 times the amount found in Types I, II, VII, and VIII specimens (1). The present study which deals with the specific polysaccharide content of pneumonic lungs lends further support to the hypothesis as given.

Method

Portions of lung removed from representative areas at autopsy were kept frozen prior to analysis. The tissues were thawed at room temperature, cut into strips, and the exudates were expressed into tubes with a forceps. Measured aliquots were then treated and analyzed for their S content in a manner similar to that described for sputum. (1) Those samples which were subjected to preliminary peptic digestion were prepared in the following manner. The expressed exudates were divided into two measured portions of 5 to 10 cc. An equal volume of acetate buffer solution and a few crystals of thymol were added to each. One tube received a small amount of crystalline pepsin and the other was left untreated. Both tubes were then corked, thoroughly shaken, and placed in the incubator at 37°C. until maximum proteolysis had occurred. This usually required from 3 to 5 days. The subsequent extractions and final analyses followed the procedure outlined for sputum (1).

RESULTS

The results of the analyses of 55 specimens of lung from 13 patients with Type I, II, VII, and VIII pneumonia are presented in Table I. The values recorded represent the average mg per cent of S in one or more lobes which showed the pathological changes indicated. The amounts of S recovered from the lungs varied from none in an uninvolved lobe to 112 mg per cent in a gray hepatized lobe with an average yield of 20.3 mg per cent for the total specimens.

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examined. While the number of cases of any one type was too small for comparative figures, more S was found in Type II and VII than in Type I and VIII cases. The yields of S from the sputum were comparable with those from the lungs, with the exception of case 4. It was also noted (Table I) that the amount of S varied with the stage of consolidation. The average figures were 33 mg per cent for the gray hepatized lobes, 21.6 mg per cent for those in the red stage, 14.9 mg per cent for the edematous and congested areas, and only 3.8 mg per cent in uninvolved lobes.

TABLE I
Specific Polysaccharide Content of Type I, II, VII, and VIII Lungs

Case No	Type	Sputum S	Gross lung pathology				
			Gray S	Red S	Edema S	Uninvolved S	Average S
		mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
1	I	—	1.6	3.4	4.7	3.2	3.2
2	I	—	10.4	1.4	5.4	7.0	6.1
3	II	13.2	12.4	—	3.5	—	8.0
4	II	3.2	23.4	—	0.7	—	12.0
5	II	94.0	112.0	17.3	0.8	—	43.4
6	VII	—	20.0	—	13.0	—	16.5
7	VII	20.0	51.0	35.0	54.0	—	46.6
8	VII	76.0	88.0	84.0	58.0	—	77.0
9	VII	—	40.0	10.0	21.0	0.0	18.0
10	VII	—	5.2	—	—	5.0	5.1
11	VIII	—	—	0.3	0.25	—	0.28
12	VIII	3.9	8.2	—	2.4	—	5.3
13	VIII	—	23.0	—	—	—	23.0
Average.		35.0	33.0	21.6	14.9	3.8	20.3

During the study it soon became evident that the S yields from the sputum in Type III pneumonia were greater than those from the lungs of the same patients as illustrated by cases 15, 16, and 17 in Table II. Inasmuch as the lungs from these patients showed reticulation in the Wright stained smears, a branching fibrin-like network of material originating in the capsules of the pneumococci (1), and as well as excess capsular carbohydrate in the "quellung" reaction, it was felt that maximal values were not being obtained. We therefore decided to subject the lung exudates to peptic digestion prior to extraction. When this was done the yields of S from the lung almost invariably exceeded those from duplicate samples treated identically except that no pepsin was added. The final S values after peptic digestion were also comparable with those from the sputum in the same patients as illustrated by cases 19, 21, and 23 in Table II.

The results of the analyses of 43 specimens from 10 patients with Type III pneumonia are presented in Table III.¹ The amounts of S recovered from the lungs varied from none in an uninvolved lobe to 5,440 mg per cent in an ede-

TABLE II

Effect of Peptic Digestion on the Specific Polysaccharide Content of Type III Lungs

Case No.	Lobes	Untreated S	Treated with pepsin S	Sputum S
		mg per cent	mg per cent	mg per cent
15	LLL	26 0	—	90 0
	LUL	16 5	—	
	RUL	15 0	—	
	RLL	3 0	—	
	RML	1 0	—	
16	LUL	36 0	—	128 0
	RUL	16 0	—	
	RML	9 2	—	
	RLL	6 0	—	
	LLL	2 0	—	
17	LUL	104 0	—	416 0
	RUL	44 0	—	
	RML	32 0	—	
	RLL	30 0	—	
	LLL	12 0	—	
19	RLL	920 0	1 210 0	1 200 0
	RML	140 0	1 200 0	
	LLL	336 0	1 160 0	
	RUL	304 0	440 0	
	LUL	0 0	—	
21	RLL	54 0	2 760 0	2 120 0
	LLL	280 0	400 0	
23	RLL	5 120 0	5 920 0	1 440 0
	RUL	5 440 0	4 800 0	
	LUL	3 600 0	4 080 0	
	LLL	560 0	2 160 0	

matous and congested lobe, with an average yield of 1,227 mg per cent for the total specimens examined. The average yield of S exceeded by 60 times the amount recovered in Type I, II, VII, and VIII cases. The sputum yields, as was expected, were also higher than the average shown in Table I. The

¹The values in cases 18 through 23 were based on data from specimens which received preliminary peptic digestion.

gray hepatized lobes contained 1,574 mg per cent with similar values of 1,536 mg per cent in the red stages, and 1,123 mg per cent in the edematous and congested areas. The amount of polysaccharide in the lungs, as in the sputum (1), appeared to increase with the degree of reticulation.

TABLE III
Specific Polysaccharide Content of Type III Lungs

Case No	Sputum S'	Gross changes in the lung					
		Reticulation	Gray S	Red S	Edema S	Uninvolved S	Average S
	mg per cent		mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
14	—	+	36 0	—	16 0	—	26 0
15	90 0	++	26 0	—	8 3	6 5	13 6
16	128 0	+	12 6	—	14 6	—	13 6
17	404 0	++++	—	28 5	74 0	22 0	41 5
18	—	++	—	544 0	—	42 0	293 0
19	1,200 0	+++	946 0	1,160 0	—	0 0	702 0
20	—	++++	1,960 0	1,080 0	—	—	1,520 0
21	2,120 0	++++	2,760 0	400 0	144 0	—	1,101 0
22	—	++++	—	2,750 0	5,440 0	1,360 0	3,180 0
23	1,440 0	++++	5,280 0	5,000 0	2,160 0	—	4,150 0
Average 897 0			1,574 0	1,536 0	1,123 0	358 0	1,227 0

TABLE IV
Specific Polysaccharide Content of the Lungs in a Mixed Type III and VII Pneumonia Presumably

Lobe	Pathology	Type III S	Type VII S
		mg per cent	mg per cent
RUL	Gray	80 0	9 0
RLL	Congested	48 0	6 0
LLL	Congested	36 0	7 0
LUL	Edema	72 0	0 0

Patient 14 in Table II is also listed as No. 6 in Table I because he was admitted to the hospital with a Type VII bacteremia which responded to treatment with sulfapyridine. He later succumbed to what appeared to be a terminal Type III pneumonia. Analyses for Type III and VII polysaccharide were made on duplicate samples of exudate from the various lobes. Although we failed to recover Type VII pneumococci from the lungs by mouse passage and only a few Type III organisms were demonstrable by direct typing, appreciable amounts of both III and VII polysaccharide were obtained, as shown in Table IV. In view of these findings, it seemed likely that the infection was a mixed one from its onset.

DISCUSSION

The specific polysaccharide recovered from the lungs and sputa (1) of patients with Type III pneumonia greatly exceeded the yields obtained in Types I, II, VII, and VIII pneumonia. We do not wish to imply that maximum lung values have been determined for each type since it is not certain that the best method of extracting S had been developed. The addition of pepsin greatly increased the recoverable S in Type III cases and it seems likely that similar treatment would also increase the yields in cases infected with other types. The average amount of S in the sputa of types other than III was 8.5 mg per cent for the total number of specimens examined and 31.1 mg per cent for the 7 cases which died (1). The latter figure compared favorably with the average yield of 20.3 mg per cent from the lungs in similar cases. The average amount of S in the sputa of Type III cases was 887 mg per cent for the total number of specimens examined and 1,643 mg per cent for the 15 cases which died (1). The latter figures compare favorably with the average yield of 1,227 mg per cent from the lungs of Type III cases. The amount of S in the sputum and lungs of fatal cases of Type III pneumonia exceeded by 53 and 60 times respectively the values obtained for the other types studied. The close relationship between the yields of S in the sputum and the amounts recovered from the lungs at autopsy suggest that the former could be utilized as a measure of the latter.

We have not attempted to correlate the polysaccharide content of sputa and lungs with the well known observations concerning S in blood and urine (2-5). However it may be remarked that Dochez and Avery (2) found polysaccharide in the blood in 4 out of 4 cases of Type III pneumonia in contrast to 4 out of 21 infections caused by Types I and II. In a recent paper Bukantz *et al.* (5) reported that S appeared in the blood most frequently in Type III cases and was detectable for long periods of time. To the authors' knowledge the only studies dealing with specific polysaccharide in the lung have been those of Nye and Harris (6). They utilized the precipitin test and estimated the S content by comparison with a known amount of polysaccharide. The maximum values which they obtained were 8,400 mg from the lungs of a Type III case and 2,300 mg from a Type I patient. For purposes of comparison we have roughly estimated the amount of S in the entire lungs of 3 cases. The calculations were based on our data and the excess weight of the lungs as compared with normal. In the first case (No. 5, Table I) it was estimated that a total of 12,500 mg of Type II S was present. The figure of 31,800 mg of Type III S was obtained for the second, and the maximum yield of 68,500 mg of Type III S was believed present in the third (Nos. 22 and 23, Table III). Approximately 750,000 mg of antibody nitrogen or 500 million units of serum would be required to neutralize 68,500 mg of polysaccharide. These findings lend further support to the opinion expressed by one of us (7) that the immunological and mechanical difficulties to which such large amounts of S would subject the

body defenses might account both for the failure of serum and the high incidence of multiple lung abscesses in Type III pneumonia

CONCLUSIONS

1 The specific polysaccharide content of pneumonic lungs in Type III pneumonia was 60 times greater than in Type I, II, VII, and VIII cases

2 The highest values were obtained from gray hepatized lobes, but the red hepatized and edematous areas also contained large quantities of S

3 Comparable yields of polysaccharide were recovered from the sputa and lungs of fatal cases

4 Preliminary peptic digest of lung exudates increased the S yields

5 The data support the hypothesis that the outcome in Type III pneumonia is related to the ability of the pneumococci to produce capsular polysaccharide

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HYPOPROTEINEMIA AS PROTECTION AGAINST MERCURIC CHLORIDE INJURY IN DOGS*

By RUSSELL L. HOLMAN M.D., AND G. L. DONNELLY, M.D.

(From the Departments of Pathology and Pharmacology University of North Carolina, Chapel Hill and the Department of Laboratories, Watts Hospital, Durham North Carolina)

PLATE 28

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During the course of experiments designed to determine whether heavy metal poisoning is influenced by altering the plasma protein level, it was found that hypoproteinemia produced by repeated plasmapheresis rendered dogs more susceptible to uranium nitrate injury (3). Whereas the majority of normal adult dogs will survive the administration of 40 mg. of uranium nitrate per kg. (4), the dose had to be reduced to 20 mg. per kg. before a dog with depleted plasma proteins survived. The question naturally arose, would increasing the concentration of the blood plasma proteins protect against heavy metal injury. When the procedure was reversed, i.e. when the dog's protein level was increased from a normal of about 6.5 per cent to 9 or 10 per cent by repeated daily injections of plasma obtained from healthy donor dogs and the dose of uranium nitrate was increased from 20 to 50 mg. per kg. (a dose that proved fatal to 13 of 14 controls (5)), no protection was demonstrable. Instead acute necrotizing arterial lesions affecting principally the large elastic arteries (aorta, pulmonary artery, endocardium of the left auricle, etc.) were found when the dogs died from "uremia" with massive necrosis of the renal tubules 8 to 17 days after the injection of uranium nitrate (6). These arterial lesions were the subject under investigation at the time the findings reported in this paper were uncovered. As was to have been expected from the general similarity of action of uranium nitrate and mercuric chloride, hyperproteinemia likewise failed to protect against mercuric chloride poisoning, similar acute necrotizing arterial lesions were found in both dogs on which this procedure was tried (7). These experiments did not prepare the authors for the finding that hypoproteinemia completely protected dogs against doses of mercuric chloride which were uniformly lethal to dogs with normal blood proteins. Yet the data presented below support this conclusion.

None of the theories about the action of mercury in therapeutic or toxic doses satisfactorily explains all the actions of this heavy metal. Mercury is usually listed as a general protoplasmic poison and many of its actions are attributable to the fact

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that as a heavy metal it can act as a protein precipitant. This concept hardly explains the curious coagulative necrosis of the proximal convoluted tubules of the kidney within 12 to 24 hours after intravenous administration and the subsequent stomatitis and gastroenteritis when presumably it would have an almost equal opportunity to precipitate every protein in the body. Nor does the explanation that it reaches an effective concentration—by reabsorption of water—in the kidneys and colon (sites of elimination) harmonize with recent chemical analyses (1) which show that most of the mercury is stored in the bones and in the liver. Inert complex salts have been postulated and some have been isolated (2) to reconcile the absence of demonstrable lesions at the sites of maximum concentration of the metal. Presumably these inert salts change as the metal is eliminated, but many gaps in our knowledge still exist.

Methods

All of the dogs used in these experiments were healthy adult mongrels ranging in weight from 4 to 19 kg and in estimated age from 1 to 3 years (pups and old dogs were excluded). They were kept in individual cages and had free access to water at all times. The diet fed the experimental animals and an equal number of the controls consisted of calves' liver (raw wet weight) 32 parts, cane sugar 25 parts, corn starch 25 parts, butter 12 parts, cod liver oil (commercial) 6 parts. Enough tomato juice was added to make a paste of which each gram contained 3 calories. 1 gm of McCollum-Simmonds salt mixture (8) and 5 gm of kaolin were thoroughly mixed with each day's diet. Essentially the diet is a low protein diet containing, on a caloric basis, 7 per cent protein (all derived from liver), 50 per cent carbohydrate, and 43 per cent fat. The diet was fed in amounts to furnish 75 calories per kg per day. Accurate records were kept of food consumption. The remaining control dogs were fed the regular kennel ration of cooked meat scraps and uncooked bones with occasional feedings of puppy dog chow. Some of the control animals received intravenous injections of trisodium citrate before and after the administration of the heavy metal. The procedure was similar to that used in published experiments with uranium nitrate (5). Since no difference could be detected clinically, chemically, or anatomically, all of these control dogs have been grouped together.

The methods for establishing the "standard hypoproteinemic state" have been published in detail (9). Briefly these consist of 1 week's fasting followed by the daily removal of approximately 20 to 25 per cent of the total blood volume of the dog and the reinjection of an equivalent amount of red blood cells suspended in a saline solution. With the dog being maintained on the standard low protein diet after the week of fasting, this procedure repeated daily 6 days per week for 2 to 3 weeks lowers the concentration of the blood plasma proteins from a normal of about 6.5 per cent to about 4 per cent and presumably exhausts all the "protein reserves."

Duplicate micro Kjeldahl analyses of total nitrogen, non-protein nitrogen (the filtrate from 10 per cent trichloroacetic acid precipitation), and albumin plus non-protein nitrogen (the filtrate from 22 per cent sodium sulfate precipitation by Howe's method) served as the basis for calculation of the plasma protein removed and blood level studies.

The mercuric chloride used in these studies was Baker's analyzed, lot 6939, and was made up in 0.1 per cent aqueous solution with distilled water. The injections

were made into the external jugular vein, each dog receiving 3.0 cc (3.0 mg) per kg. About 5 minutes were required for the injections. No immediate reactions such as vomiting were noted.

The experimental animals were kept under close observation, were sacrificed with ether if they were obviously moribund, and were necropsied promptly after death. Routine sections, taken from all organs and from many of the tissues, were fixed in 4 per cent solution of formaldehyde and Zenker's solution. In some of the control dogs only sections from the liver and from both kidneys were studied. The tissues fixed in Zenker's solution were embedded in paraffin, sectioned at 6 microns, and stained routinely with hematoxylin and eosin. Appropriate special stains were used in selected cases.

EXPERIMENTAL OBSERVATIONS

The first procedure was to establish the minimum lethal dose of mercuric chloride. The literature on this subject is conflicting. The most definite statements are based on administration by the intravenous route. Sansum (10) states: "The minimum uniformly lethal intravenous dose of mercuric chloride in dogs was found to be 4 mg per kg body weight (1:1000 solution injected uniformly during 15-60 minutes)," and the efficacy of this dosage has been confirmed (11, 12). Havill, Lichty, and Whipple (13) found this dose too large and stated that the minimum lethal dose for dogs is 1.5 to 2.0 mg per kg. As a starting point we decided to use 3.0 mg per kg, and as can be seen from Table I this dose proved uniformly fatal to 12 dogs with normal blood proteins in 4 to 11 days. All of the dogs became sick, refused food, and exhibited marked nitrogen retention. The kidneys of all of these dogs showed the classical picture of massive necrosis of the epithelium lining the proximal convoluted tubules (Fig. 1). Calcification was marked as early as 4 days after the injection, and there was only slight evidence of regeneration as late as 11 days. Practically all of these dogs showed extensive necrotizing stomatitis, and several had acute inflammatory lesions in the stomach and colon. The three dogs which were fed the standard diet did not differ from those which were fed the kennel diet.

In sharp contrast to these results the three dogs reduced to the standard hypoproteinemic state by bleeding and return of the washed red blood cells suspended in saline showed practically no effects following the administration of the same dose of the same standard solution of mercuric chloride (Table II). None of these dogs became sick, none refused food for a single day, none showed elevation of the blood non protein nitrogen, and the kidneys both in the gross and microscopically appeared normal when they were examined 8, 14, and 45 days after the injection of the heavy metal (Figs. 2 to 4). None of the dogs had any "hemolytic reactions" during the period of plasmapheresis, and none showed any pigment in the kidney on histological study. In other words, there is no evidence that the protective action of hypoproteinemia was in any way related to hemoglobin in the manner demonstrated by Havill, Lichty, and Whipple.

TABLE I

Intravenous Administration of 30 Mg of Mercuric Chloride per Kg Uniformly Fatal to Dogs Regardless of Diet

Dog No	Diet	Body weight	HgCl ₂	Death after	Terminal N.P.N	Kidney*	
						Necrosis	Calcification
		kg	mg/kg	days	mg/100 cc		
41-88	Kennel	17 5	3 0	4	330	+++	+†
41-96	"	12 0	3 0	11	692	+++	+++
41-97	"	14 8	2 0	9	531	+++	+†
B-30	"	18 4	3 0	5	207§	+++	+++
B-31	"	19 2	3 0	10	320	+++	+++
B-32	"	8 5	3 0	5	295§	+++	+
B-33	"	7 5	3 0	5	220§	+++	+++
B-34	"	9 0	3 0	4	230¶	+++	+++
B-35	"	8 9	3 0	4	210¶	+++	++
42-2	Standard	5 7	3 0	6	436	+++	+
42-3	"	5 3	3 0	4	289	+++	+++
42-4	"	3 5	3 0	5	374	+++	+++

* The extent of the lesion has been graded as follows + slight, ++ moderate, +++ marked

† Slight evidence of regeneration

§ N P N 2 days before death.

|| " 3 " " "

¶ " 1 day " "

TABLE II

*Intravenous Administration of 30 Mg of Mercuric Chloride per Kg without Effect in "Standard Hypoproteinemic Dogs"**

Dog No	Body weight	No of exchanges	Total plasma removed	Plasma protein concentration		HgCl ₂	Highest N.P.N	Sacrificed after	Kidney
				At start	At end				
	kg		cc	gm/100 cc	gm/100 cc	mg/kg		days	
40-81	5 0	12	1078	7 3	4 6	3 0	20†	45	Normal
41-90	7 4	18	1956	8 2	4 1	3 0	31	14	"
41-95	9 5	17	1949	6 7	4 3	3 0	22†	8	"

* All three dogs maintained on standard diet.

† It is worth recording that the N P N actually fell to 14 mg per 100 cc. on the 10th day after the mercury in the case of dog 40-81, and to 16 mg per 100 cc. on the 7th day in the case of dog 41-95. No such fall was observed in dog 41-90.

(13) The only findings that could be attributed to the mercury were small focal areas of stomatitis on the mucous membranes of the upper lip. In dog 41-95 the process was acute, in dog 41-90 the process was healing, and in dog 40-81 the process had healed.

A limited number of experiments have failed to demonstrate any protective action of plasmapheresis *after* the injection of mercuric chloride. The following protocol is illustrative of the results obtained—

Dog 41 99 Male mongrel estimated age 1 year Kennel diet

Nov 27, 1941 Weight 5.58 kg Control blood studies hematocrit 35 per cent, non-protein nitrogen 33 mg per 100 cc., blood plasma proteins 7.1 gm. per 100 cc. albumin globulin ratio 0.83 16.74 cc. of 0.1 per cent solution of mercuric chloride injected into external jugular vein 2:30–2:35 p.m., followed by 3 plasmaphereses as follows 1st exchange (155 cc. bled 60 cc. packed red blood cells—obtained from donor—suspended in saline injected) completed at 2:50 p.m. 2nd exchange (125 cc. bled, 50 cc. packed cells injected) completed at 4:30 p.m. 3rd exchange (80 cc. bled 50 cc. packed cells injected) completed at 6:15 p.m.

Nov 28, 1941 Weight 5.20 kg Some retching and vomiting this a.m. 4th exchange (160 cc. bled, 72 cc. packed cells injected) completed at 11:00 a.m., 5th exchange (125 cc. bled 54 cc. packed cells injected) completed at 3:00 p.m.

Nov 29, 1941 Weight 5.00 kg No food consumed since injection of mercury Obviously sick this a.m. Blood studies (sample at 8:45 a.m.) plasma faintly icteric, hematocrit 35 per cent non-protein nitrogen 206 mg per 100 cc. blood plasma proteins 5.7 gm. per 100 cc. albumin globulin ratio 0.97 Died at 1:00 p.m.

Necropsy revealed extensive necrosis and calcification of the epithelium lining the proximal convoluted tubules (Fig 5) moderate fatty change in the liver with early central necrosis and focal hemorrhagic necrosis in the mucosa of the colon

Replacement of about 125 per cent (5 exchanges totalling 645 cc.) of the total circulating blood volume with washed red blood cells suspended in saline within 24 hours after the injection of mercuric chloride failed to afford any protection against the heavy metal. Rather the typical coagulative necrosis and calcification of the renal epithelium appeared to be augmented and accelerated by the plasmaphereses. Similar results were obtained by Haskell, Hamilton, and Henderson (11) following “exsanguination transfusion” in dogs after the intravenous administration of 4.0 mg of mercuric chloride per kg

DISCUSSION

The authors have no satisfactory explanation for the finding that standard lethal doses of mercuric chloride produce little or no effect in hypoproteinemic dogs. It would appear that hypoproteinemia cannot be used to combat mercuric chloride poisoning which is already under way. This is in harmony with the results of Haskell, Hamilton, and Henderson (11) who found “exsanguination-transfusion” ineffective in the treatment of mercuric chloride poisoning. Apparently the hypoproteinemia must *precede* the administration of the mercury. Much work remains to be done before the degree and duration of hypoproteinemia that must exist for this protective action—also the limit of this protective action—can be established. One possible therapeutic benefit

might result from the effort—mercurial diuretics such as salyrgan and novasurol might be used with greater assurance for the patient's safety in cases of nephrosis and nutritional edema

The finding that hypoproteinemia protects against mercuric chloride injury, contrasts sharply with the results of similar experiments with uranium nitrate in which it has been shown that the hypoproteinemic state renders the animals more susceptible to uranium (3). The results with mercury, though based on a small number of animals, are uniform and very sharp, and warrant the hypothesis that the mode of action of the two heavy metals is different. At least a tool is presented by which this aspect of the subject can be investigated.

Another fact that seems to be clearly established is that reversal of the procedure—hyperproteinemia by plasma injections instead of hypoproteinemia by plasmapheresis—failed to demonstrate any difference between the two heavy metals. There was no protective action against either heavy metal and similar acute necrotizing arterial lesions were found after the administration of both uranium (6) and mercury (7).

While "normal" and "hyperproteinemic" dogs react similarly to uranium and mercury, a striking difference in the action of the two heavy metals is demonstrable in standard hypoproteinemic dogs with "reserve stores" of protein exhausted. Following uranium the hypoproteinemic dog quits eating, develops acidosis and marked nitrogen retention, and dies 6 to 17 days later with massive necrosis of the proximal convoluted tubular epithelium. The only difference between the hypoproteinemic and the normal dog is that in the former less uranium is required to produce these classical results. Following the intravenous administration of a dose of mercury, uniformly fatal to normal dogs, the hypoproteinemic dog fails to show any evidence of illness. Appetite and body weight were maintained in our animals, there was no nitrogen retention, and the kidneys both in the gross and histologically appeared normal when the dogs were sacrificed 8, 14, and 45 days later. There is no evidence—such as calcification, mitoses, or basophilic "flattened epithelium," emphasized by MacNider (12)—that the kidneys of these dogs would have shown any change if examined earlier than 8 days. And with the exception of small localized areas of healing stomatitis on the mucous membrane of the upper lips none of the other organs or tissues showed any changes that could be attributed to the heavy metal.

Four or five mechanisms come to mind that might explain findings, but the available data do not allow a satisfactory evaluation of any of them. The simplest purely theoretical explanation for the observed phenomena is that the establishment of the hypoproteinemic state depletes something (presumably fabricated or concentrated in the kidney cortex) and this substance is not available to be acted on by the heavy metal. Still other possibilities are (1) The hypoproteinemic state fails to "hold" the mercury protenate (this hypothesis involves differential solubilities in different concentrations of

proteins), (2) The acid base balance may be upset and the pH of the medium so changed that it favors a shift from "toxic" to "non toxic" mercurial compounds, (3) blood calcium may be altered and if mercury "follows" calcium as Young, Taylor, and Merritt (1) have shown, this may account for the failure of mercury to reach "effective concentration," (4) the reducing powers of the blood—e.g. as that of the sulfhydryl groups—may be altered as postulated by Miller and Whipple (14, 15) to explain the increased susceptibility of standard hypoproteinemic dogs to chloroform anesthesia and the protective action of methionine and cystine against this increased susceptibility. Further speculation seems inappropriate at this time: the data had best rest on their own merits.

SUMMARY

Twelve control dogs receiving a single intravenous injection of mercuric chloride, 3.0 mg per kg, all died within 4 to 11 days afterwards with marked nitrogen retention and extensive necrosis and calcification of the epithelium lining the proximal convoluted tubules.

Three dogs of comparable age and weight were reduced to a standard hypoproteinemic state by repeated plasmapheresis. Each dog then received the same dose of mercuric chloride as the controls. None of these dogs became sick, none showed any elevation of non-protein nitrogen, and the kidneys—both in the gross and histologically—appeared normal when they were examined 8 to 45 days later.

As tested thus far intensive plasmapheresis following the injection of mercuric chloride has been without effect in preventing the classical changes of mercuric chloride injury observed in the control dogs.

The simplest explanation for these phenomena is that mercuric chloride acts on a more or less specific substance (presumably fabricated or concentrated in the renal cortex) which is depleted in the standard hypoproteinemic state. Other possibilities are mentioned.

These findings are in sharp contrast to the results of similar experiments with uranium nitrate. The hypoproteinemic state appears to render the animals more susceptible to uranium injury (3). This probably indicates that the mode of action of the two heavy metals is different.

CONCLUSION

Lethal doses of mercuric chloride produce little or no effect in standard hypoproteinemic dogs.

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EXPLANATION OF PLATE 28

All sections of kidney fixed in Zenker's fluid and stained with hematoxylin and eosin

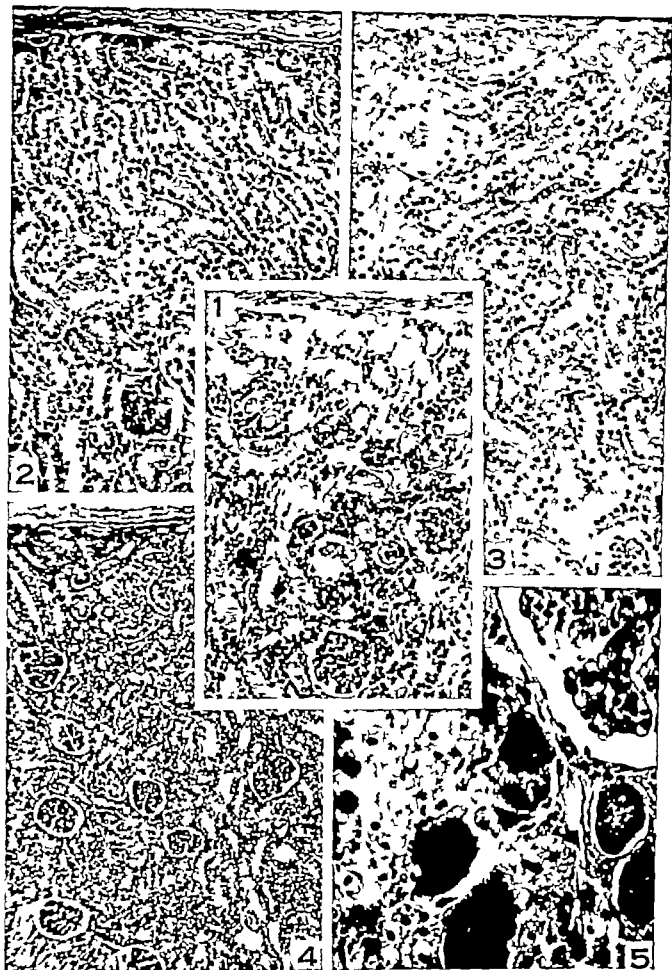
FIG 1 Dog 42-2 Normal dog on "standard diet" for 6 weeks 6 days after mercuric chloride Typical coagulative necrosis of proximal convoluted tubules $\times 145$

FIG 2 Dog 40 81 Hypoproteinemic dog Absence of any evidence of injury 45 days after mercuric chloride $\times 148$

FIG 3 Dog 41-95 Hypoproteinemic dog Absence of any evidence of injury 8 days after mercuric chloride $\times 170$

FIG 4 Dog 41-90 Hypoproteinemic dog Absence of any evidence of injury 14 days after mercuric chloride $\times 88$

FIG 5 Dog 41-99 Normal dog on kennel diet Mercuric chloride followed by intensive plasmapheresis Extensive necrosis with marked calcium deposition in proximal convoluted tubules 2 days after heavy metal $\times 360$



LOW PROTEIN DIET AUGMENTS HYPERPROTEINEMIA PRODUCED BY REPEATED INJECTIONS OF HOMOLOGOUS PLASMA

EVIDENCE FOR A DYNAMIC EQUILIBRIUM BETWEEN FOOD, PLASMA,
AND TISSUE PROTEINS*

By RUSSELL L. HOLMAN M.D

*(From the Department of Pathology, University of North Carolina, Chapel Hill and the
Department of Laboratories, Watts Hospital Durham North Carolina)*

(Received for publication, August 3, 1942)

During recent years the use of plasma transfusions has become widespread and increasingly so of late as a result of war conditions. More and more the need for really large amounts of plasma in treating certain types of cases is being appreciated. The data presented in this paper should be of interest to those engaged in studying the quantitative aspects of such therapy. Further these data have certain theoretical implications that bear upon the more general problems of plasma protein formation and protein metabolism.

Hyperproteinemia can regularly be produced in dogs, and presumably in other species of animals, by repeated injections of plasma obtained from homologous donors. Almost none of the injected protein escapes in the urine as protein, nor is it eliminated quantitatively as increased urinary or fecal N_2 . While no direct measurements have been made in dogs, Addis' results following intraperitoneal injections of serum in rats (1) indicate that the protein content of all of the viscera and tissues is increased. The greatest increment is in the serum, next in the liver, but all organs and tissues show a definite increase in protein content.

Practically all of the experiments to date (2-6) have been carried out with the recipient of the plasma or serum injections maintained in a fasting state or receiving only sugar, or sugar and fat, by mouth. *A priori* one would expect that plasma injections in an animal maintained on a full diet or a high protein diet would yield summation effects and result in a more marked hyperproteinemia. In the experiments reported below this does not happen, in fact, a significantly higher hyperproteinemia is produced when the animals are maintained on a low protein diet than when a high protein diet is given. This seeming paradox is open to a number of interpretations but all of them seem to imply an equilibrium between food, plasma, and tissue proteins.

* This work has been aided by a grant from The John and Mary R. Markle Foundation.

Methods

All of the dogs were healthy adult mongrels. Small dogs (about 50 kilos) were chosen so that increase of the plasma protein level could be accomplished without using too great quantities of blood. They were kept in individual cages and had free access to water at all times. One group was fed a high protein diet, the other group a low protein diet.

The high protein diet consisted of lean beef—25 gm per kg per day—to which 1 gm of the salt mixture (7) was added.

The low protein diet consisted of calves' liver (raw wet weight) 32 parts, cane sugar 25 parts, corn starch 25 parts, butter 12 parts, and cod liver oil 6 parts. 1 gm of salt mixture (7) and 5 gm. of kaolin were thoroughly mixed with each day's diet. Enough tomato juice was added to make a pasty mixture of which each gram contained 3 calories. The diet was fed in amounts to furnish 75 calories per kg per day. Essentially this diet is a low protein diet with 7 per cent of its caloric value derived from protein, 50 per cent from carbohydrate, and 43 per cent from fat.

All of the dogs in both groups consumed 100 per cent of the diet each day.

The methods used in making the dogs hyperproteinemic have been published in detail (2, 8). Briefly these consisted of bleeding a donor—a sufficient number of large dogs (15 to 25 kg) were used so that anemia did not develop—about 200 cc into a flask containing 2.5 cc. of a saturated solution of trisodium citrate, centrifuging the citrated blood in 100 cc centrifuge tubes at 3000 R P M for 30 minutes, withdrawing the plasma (usually 100 to 110 cc) with suction, warming it to 40°C, and injecting it into one of the external jugular veins (about 10 minutes being required for each injection). This procedure was repeated daily, 6 days per week, for 3 to 4 weeks.

Duplicate micro Kjeldahl analyses of total N, N P N (the filtrate from 10 per cent trichloroacetic acid precipitation) and albumin plus N P N (the filtrate from 22 per cent sodium sulfate precipitation by Howe's method) served as the basis for calculating the blood level studies. All of the recorded studies were made on hematocrit samples (using 2.0 cc. of 1.4 per cent sodium oxalate and 10 to 13 cc of blood), and the "final samples" were taken at least 18 hours after the last injection of donor's plasma.

EXPERIMENTAL OBSERVATIONS

The experimental data are summarized in Tables I and II.

In dogs consuming a high protein diet (Table I) the intravenous injection of plasma obtained from healthy donor dogs, amounting in all to five or six times the quantity of plasma protein in circulation at the start, resulted in only a moderate increase in the concentration of the blood plasma proteins. The average increase was 20 per cent. As would be expected, there was a fall in the hematocrit reading but this was not great—ranging from about 5 per cent in dog 35-693 to about 28 per cent in dog 40-60 and averaging 18 per cent. "Final" blood and plasma volume studies made in isolated cases in this series agree with previous observations (2, 3) that these are increased

only about 15 per cent, and this increase—which is almost entirely in the plasma volume—accounts in part at least for this fall in hematocrit value. There were no significant changes in the albumin globulin ratio, plasma N P N, or in body weight.

TABLE I
Plasma Injections in Dogs on High Protein Diet

Dog No	Body weight		No of injections	Total amount injected	Plasma protein concentration		Albumin/globulin		N.P.N		Hematocrit reading	
	I	F ‡			I	F	I	F	I	F	I	F
	kg	kg		cc	gm /100 cc				mg /100 cc.		per cent	
35-693	12 7	12 7	19 in 24 days	4355	6 6	8 2			27	27	41	39
40-59	6 3	5 8	18 21 "	1595	7 5	8 9	1 2	1 3	36	36	58	44
40-60	6 5	6 3	24 28	2665	7 2	8 7	1 7	1 4	30	30	57	41
40-67	4 8	4 3	20 24	2080	7 2	8 3	0 9	0 8	40	32	53	39

* Initial value—before first plasma injection.

‡ Final value—18 to 24 hours after last plasma injection.

TABLE II
Plasma Injections in Dogs on Low Protein Diet

Dog No	Body weight		No of injections	Total amount injected	Plasma protein concentration		Albumin/globulin		N.P.N		Hematocrit reading	
	I	F ‡			I	F	I	F	I	F	I	F
	kg	kg		cc	gm /100 cc.				mg /100 cc.		per cent	
39-28	6 7	5 1	24 in 28 days	2225	7 2	9 9	1 3	0 7	41	23	36	32
39-34	4 5	4 4	22 28	2625	6 5	9 4			30	41	43	40
39-40	6 3	6 5	17 " 21 "	2010	7 0	8 8	1 3	1 0	33	32	51	42
39-45	7 1	7 3	22 26	2210	7 1	9 2	1 2	0 9	36	31	54	43
40-50	4 7	4 8	24 28	1880	6 6	10 0	1 9	0 9	41	25	55	49
40-63	8 1	9 2	24 " 28 "	2960	6 0	8 0	1 2	1 1	28	28	48	36
40-80	5 7	5 8	18 21	1840	6 7	9 4	1 3	1 0	31	24	48	38

* Initial value—before first plasma injection

‡ Final value—18 to 24 hours after last plasma injection.

In dogs consuming a low protein diet (Table II) the intravenous injection of comparable amounts of plasma—obtained in most instances from the same donor dogs—resulted in a more marked increase in the concentration of the plasma proteins. The average increase was 40 per cent, or twice as great as in the group of dogs maintained on high protein diet. Changes in the hematocrit readings were in the same direction and of the same order of magnitude in

both groups In the group on the low protein diet (Table II) the albumin globulin ratio fell in every instance and in some of the dogs (e g 40-50) this change was marked The average reduction in this ratio in the six dogs on which it was determined was about 30 per cent There were no significant changes in plasma N P N or in body weight

Following cessation of the daily injections, the plasma protein level returned to approximately normal in about 2 weeks regardless of diet

DISCUSSION

A *dynamic equilibrium* between food, plasma, and tissue proteins was postulated by Holman, Mahoney, and Whipple in 1934 (2) The evidence which has accumulated since that time has lent support to this view The subject has recently been reviewed by Madden and Whipple (9) and by Whipple (10)

Briefly stated this concept implies that food protein, absorbed from the gastrointestinal tract as amino acids, can be fabricated into units (or aggregates) in one portion of the body for utilization in another part of the body During transport these units (or aggregates) constitute part of the plasma proteins It is probable that a large portion of this synthesis takes place in the liver, but other tissues are undoubtedly involved, e g antibody (globulin) formation by lymph nodes and insulin production by the islets of Langerhans, and it is not improbable that much of this equilibrium is maintained in the blood which as an organ is about four times the size of the liver This concept also implies that neither the capillary endothelium nor the cell membrane is impermeable to these units or aggregates which by the usually employed methods are classed as proteins Direct measurement of lymph protein in all parts of the body and the rapid rate of restoration of plasma protein following acute severe hemorrhage or plasmapheresis lend support to both of these assumptions This view in no way invalidates the Starling hypothesis, but merely adds an adaptive mechanism that makes it more dynamic

At first glance it might seem that the data presented in this paper do not support the hypothesis of an equilibrium It might be contended that the more protein going into the body by whatever route, the greater should be the concentration in the plasma and in the tissues Within narrow limits this is true, for by high or low protein feeding the concentration of plasma protein can be raised or lowered by 5 to 10 per cent of the normal It must be remembered, however, that the nitrogen balance can be established with widely varying intakes, and that under these wide variations in the intake the body holds tenaciously to a rather limited zone of concentration of plasma protein (5.5 per cent to 7.0 per cent) Marked reductions in diet or great physical removals (plasmaphereses) must be carried out in order to lower significantly the plasma protein concentration The same is true of hyperproteinemia—relatively large quantities of donor's plasma totalling several times the amount

actually in circulation at the start of the experiment must be injected before significant elevations in the plasma protein concentration are effected. During the first week of such injections the increment in the concentration of the plasma protein is slight, and it is only during the later weeks that significant amounts of the injected protein pile up in the circulation. These amounts are greater with low protein feeding, presumably because less of the necessary "chemicals" to maintain the normal equilibrium is supplied. High protein feeding on the other hand maintains the normal equilibrium better by supplying more of these necessary "chemicals."

Qualitative differences in the utilization of the injected protein under varying conditions of dietary protein are indicated by the fact that in the animals maintained on a low protein diet the albumin globulin ratio is decreased, whereas there is no significant change in this ratio in the animals maintained on a high protein diet. Most of the difference in the extent of the hyperproteinemia in the two groups can be accounted for by the greater increase in globulin in the group maintained on a low protein diet. It could be argued that the more marked hyperproteinemia in this group is necessary to maintain the same osmotic pressure relationships. This is a teleological argument and is not supported by available data, for albumin is not decreased and there is no need for a further increment in globulin. Rather the data point to an upset in the normal equilibrium with the accumulation of an excess of globulin. Whatever the true explanation happens to be, it seems definite from the experiments here reported that the hyperproteinemia following repeated plasma injections is *greater* in animals maintained on a *low* protein diet than in animals maintained on a *high* protein diet.

The promptness with which the plasma proteins return to normal level after cessation of injections or withdrawals despite high or low protein feeding serves to emphasize the abnormality that must be present in certain cases of Bright's disease in which "optimum" feeding fails to influence materially the hypoproteinemia, and in some cases of multiple myeloma in which extreme hyperglobulinemia persists even during inanition and fasting. The comparative rareness of these abnormalities serves in turn to emphasize the stability of the normal mechanism which must be of the nature of an equilibrium between food, plasma, and tissue proteins.

The data in this paper do not embrace the tissue proteins except indirectly in the figures on body weight. In previous publications (2-4, 8, 9) the part played by the tissue proteins in this equilibrium has been discussed.

The experimental findings in this paper and the general thesis of an equilibrium have received partial confirmation by other workers using the opposite approach to the problem, namely treatment of hypoproteinemia occurring in human cases of "nephrosis" or induced in experimental animals by plasmapheresis or extremely low protein feeding. Liu and Chu (11), Keutmann

and Bassett (12), and Farr (13) have all found an "optimum" intake of about 30 gm of protein per kg per day. Greater intake resulted in less retention. Whipple and his coworkers (10) found, "In general, food proteins are better used when given alone and in moderate amounts. Larger protein intake yields a lower per cent return of plasma protein."

The "ideal" protein to combat hypoproteinemia has not yet been defined, but it is reasonable to predict on the basis of the findings reported in this paper that when it is defined quantitative as well as qualitative factors will play an important part in the utilization of that protein. It is possible that this "ideal" protein may prove to be a more or less specific substance that controls the normal equilibrium between food, plasma, and tissue proteins. Possibly the repeatedly confirmed observation (9, 14, 15) that serum protein is the most potent of all the proteins that have been tested thus far for combating hypoproteinemia means that more of this hypothetical substance is present in serum.

SUMMARY

1 In 4 dogs maintained on a *high* protein diet (lean meat) repeated intravenous injections of plasma obtained from healthy donor dogs (18 to 24 injections during the course of 3 to 4 weeks, totalling 1595 to 4355 cc—averaging 1800 cc when figured on the basis of a 5 kg dog) resulted in a mean increase in the plasma protein concentration of 20 per cent (from 7.1 per cent to 8.5 per cent).

2 In 7 dogs maintained on a *low* protein diet (only 7 per cent of total caloric value derived from protein) almost identical injections of donor's plasma caused an average increase in the plasma protein concentration of 40 per cent (from 6.7 per cent to 9.4 per cent).

3 The albumin globulin ratio in the group on the low protein diet showed an average fall of 30 per cent (from 1.4 to 0.9) while in the group on the high protein diet the change in this ratio was insignificant (from 1.3 to 1.2).

4 In all dogs in both groups there was a consistent fall in the hematocrit value of about 15 to 20 per cent (from 49 to 40, or 18 per cent) which can be explained in part at least by the increase in plasma volume of about 15 per cent.

5 There were no significant changes in body weight or in plasma N P N.

CONCLUSIONS

1 Hyperproteinemia produced by repeated daily injections of homologous plasma is more marked in dogs maintained on a low protein diet than it is when comparable amounts of plasma are injected into dogs maintained on a high protein diet.

2 This seeming paradox is interpreted as additional evidence for a dynamic equilibrium between food, plasma, and tissue proteins.

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COAGULATION AND LIQUEFACTION OF SEMEN*

PROTEOLYTIC ENZYMES AND CITRATE IN PROSTATIC FLUID

BY CHARLES HUGGINS M.D., AND WILLIAM NEAL M.D.

(From the Department of Surgery of The University of Chicago, Chicago)

(Received for publication, August 3, 1942)

The semen of man and animals is delivered from the urethra in a liquid state but differs thereafter in its properties in three principal ways. In the guinea pig it becomes an elastic solid which is maintained in this condition both *in vitro* and in the vagina for many days. The semen of the dog remains liquid. The ejaculate of normal man solidifies but then liquefies in a few minutes at room temperature.

The solidification of the semen of the guinea pig is due to the action of an enzyme, vesiculase (1, 2) derived from a special region of the prostate (3) on the proteins of the seminal vesicle. No experiments have been reported dealing with the solid liquid phases of human semen or with the persistence of the liquid state of canine semen, matters with which the present paper is concerned.

Our first observation was that a mixture of human semen with blood underwent coagulation but soon liquefied. Most of the present experiments deal with various phases of the effects of semen and its components on blood and its clotting constituents.

Methods

Human semen was obtained by manual ejaculation. Following liquefaction, it was centrifuged and the supernatant fluid used immediately for testing. Canine semen was obtained by pilocarpine stimulation following the prostatic isolation procedure of Huggins, Masana, Eichelberger, and Wharton (4); this fluid was passed through a Seitz filter to obtain a sterile filtrate free from cells.

Fresh blood and citrated plasma were obtained from various species. The plasma was procured by centrifugalization of blood drawn into a solution of sodium citrate 5 per cent, of which 1 cc. was used for each 9 cc. of blood. It was recalcified with a solution of CaCl_2 1.5 per cent, of which 0.33 cc. was used for each cubic centimeter of plasma.

Testing for lytic activity was carried out by mixing 1 cc. of blood or plasma with 1 cc. of semen or other fluid under examination, either undiluted or diluted to 1 cc. with saline. After mixing, the tubes were stoppered and placed in a water bath at 37°C. and

* This investigation was supported by grants from the Committee for Research in Problems of Sex, the National Research Council, and from the National Committee on Maternal Health.

the times of coagulation and of subsequent liquefaction, when it occurred, were noted. In stating the dilutions in this paper the actual content of secretion in 1 cc. is given, saline refers to an aqueous solution of NaCl, 0.15 molar in concentration, and incubation refers to 37°C. Where citrated plasma was used the specified concentration of calcium chloride was added according to two plans, either the plasma was recalcified immediately after adding the fluid to be tested, or after a delay of minutes to hours to permit a prolonged contact, in the liquid state, of the plasma and the fluid under test.

As control, saliva, bile, cerebrospinal and spermatocele fluids were substituted for semen and the effects on the clotting and liquefaction of blood noted.

In testing the effect of the lytic factors in semen on the clotting constituents of human blood, fibrinogen, thrombin, and thromboplastin were prepared from beef plasma and lung by the method of Smith, Warner, and Brinkhous (5) and prothrombin by the technique of Seegers and colleagues (6).

Citrate analyses were made by the method of Pucher, Sherman, and Vickery (7) and throughout this paper the term, citrate, refers to the combined amount of citric acid and its salts as determined by this method. Trypsin was determined by the method of Anson (8).

The opacity developing coincident with clot formation induced by recalcification of citrated plasma, and the effect of semen upon this density were studied in an Evelyn photoelectric colorimeter. The colorimeter was mounted in an incubator at 37°C and all of the solutions were brought to this temperature before using. A 6600 Ångström light filter was used. The total volume in each colorimeter tube was 13.5 cc. distributed as follows —

5 cc. of plasma + 2 cc. of semen (saline in the control tubes) + 5 cc. of saline + 1.5 cc. of CaCl_2

Following recalcification, readings of the galvanometer were recorded each 12 seconds until a steady state had been reached following coagulation, or until it was evident that clotting was not occurring.

RESULTS

The Phenomenon of Spontaneous Lysis of Human Semen — The semen of normal men after ejaculation forms a solid mass and the container may be inverted without losing any of the material, within a few minutes softening and partial liquefaction is observed and within 15 minutes the ejaculate is in a liquid state save for a few sago-like particles, derived from the seminal vesicles, which remain for more than 1 hour.

The lytic phenomenon may be observed well through the microscope. In a typical example, at 3 minutes after ejaculation a thin section appeared as many interlaced bundles of long, clearly defined, parallel, refractile fibres, at 4 minutes the fibres appeared swollen, at 5 minutes the regularity had disappeared and the fibres were arranged haphazardly, at 6 minutes there was large scale liquefaction with movement visible as many of the fibres were rapidly breaking up. No fibres were seen 8 minutes after ejaculation.

In a man with hypogonadism due to undescended testes the semen did not

clot, following intramuscular injection of testosterone propionate, 25 mg daily for 15 days, normal clotting was observed

The Effect of Semen and Prostatic Fluid on the Coagulation Time of Blood—The clotting time of whole blood, 1 cc. mixed with saline, 1 cc., in 144 instances ranged from 3 to 10 minutes.

(a) *Human semen* Two effects were observed, shortening and prolongation of the coagulation time of whole blood. Of 23 seminal specimens tested against whole human blood, 11 prolonged the coagulation time, while 12 caused a decrease of the clotting time as compared to the control saline mixtures. In the group in which a mixture of semen lessened the time of coagulation, the effect was evident with as little semen as 0.025 cc. (Table I) This

TABLE I

Effect of Semen and Other Human Secretions on the Coagulation Time of Human Blood
1 cc. of whole blood + 1 cc. of secretion diluted secretion, or saline.

Nature of secretion	Coagulation time								
	Amount of secretion in test								
	None	1 cc.	0.5 cc.	0.1 cc.	0.05 cc.	0.033 cc.	0.025 cc.	0.0166 cc.	0.0125 cc.
	min.	min.	min.	min.	min.	min.	min.	min.	min.
Semen, group 1	8	2	3	2	2	3	3	—	5
Semen group 2	5	30	14	6	5	5	5	5	5
Prostatic fluid	5	0	0	0	19	11	7	7	6
Prostatic fluid heated	5	0	0	0	22	—	—	—	—
Prostatic fluid and CaCl_2 *	5	3	3	4	—	—	—	—	—
Seminal vesicle secretion	5	—	3	4	4	5	5	5	—
Hydrocele fluid	5	2½	2½	3	3	3	—	—	—
Saliva	5	3	2	3	—	—	—	—	—

* Calcium chloride, 1.5 per cent, 0.33 cc.

lessened coagulation time is due to thromboplastic substances in appreciable amounts. In the group with prolonged clotting with semen in 1 cc. amounts no, or slight clot, was observed in 30 minutes, in this group, semen in 0.2 or 0.1 cc. amounts often lengthened clotting time to, 15 or more minutes.

(b) *Human prostatic fluid* All specimens of undiluted fluid tested (from 18 men) inhibited the clotting of human blood. With prostatic fluid 0.1 cc., clotting occurred in 14 to 50 minutes in 5 specimens, while no clot was observed in 2 hours in 13 observations. The coagulation time of blood mixed with prostatic fluid 0.02 cc. was approximately normal.

Coagulation of the blood was always induced by adding calcium chloride, 1.5 per cent, 0.33 cc. to prostatic fluid, 1 cc. In 5 cases incoagulability, or greatly prolonged clotting time, was abolished and a clotting time shorter than normal supervened, in 3 cases the coagulation time was greatly decreased

but still remained longer than the normal control. In all cases in which semen caused a prolonged clotting of admixed blood, the addition of the specified amount of calcium chloride abolished the delay and coagulation occurred more rapidly than in the control saline-blood mixture.

(c) *Dog prostatic fluid* Of 40 specimens of human blood, 1 cc, mixed with prostatic fluid, 1 cc, the coagulation time was normal or shortened in 32 cases. In 4 cases clotting did not occur while only slight clot was observed in 13 to 20 minutes with the fluid of 4 dogs in this group. In contrast to the system, human prostatic secretion-blood, the addition of calcium ions did not initiate or hasten clotting and the delay was due to a different mechanism, namely, the disappearance of fibrinogen as it is discussed below.

Citrate Is the Cause of Prolonged Coagulation Time of Mixtures of Blood and Human Prostatic Fluid or Semen—It was found that human prostatic fluid heated in a water bath at 100°C for 30 minutes still delayed blood coagulation, while the delay was abolished by adding calcium ions (Table I). Large amounts of citrate in the prostatic fluid were discovered by Scherstén (9) and the finding was confirmed by Dickens (10). We likewise observed large amounts of this acid, obtaining the following values for citrate in each 100 cc, 15 specimens of human semen, 140 to 637 mg, 9 samples of human prostatic fluid, 480 to 2688 mg, 20 lots of dog semen, 0 to 2.6 mg, 2 analyses of human seminal vesicle secretion, 15 and 22 mg. While Huggins, Scott, and Heinen (11) found a high calcium content in human semen (6 millimols per liter) and in human prostatic fluid (30 mM per liter) the concentration of citrate in prostatic fluid and some seminal specimens is so high that the calcium ions are reduced preventing thrombin formation and blood coagulation. It seems reasonable to conclude that the adverse effect of human prostatic fluid on blood coagulation is a citrate effect.

Clotting Constituents of Human Semen—The principal clotting constituents of blood were prepared from beef plasma and lung. In order to determine the presence of these or similar substances in semen, various combinations of them were placed in contact with human semen which had become liquid and the presence or absence of coagulation and the clotting time determined. Differences were observed depending on whether calcium ions were added to induce clotting immediately after adding semen or after preliminary incubation of semen with the clotting fractions of blood in the liquid state.

(a) *Immediate induction of clotting* Human semen was found capable of substituting for fibrinogen and thromboplastin but not for prothrombin in inducing clotting (Table II, lines 1-6). The clot formed in the absence of added fibrinogen, was not firm, indicating that the fibrinogen content of semen was less than that of the prepared extract. Goldblatt (30) discovered that human semen contained thromboplastin.

(b) *Delayed induction of clotting* Incubation of prothrombin with semen

both for 40 minutes and 18 hours decreased the coagulation time on adding fibrinogen, thromboplastin, and calcium ions (Table II, lines 7-10). Incubation of fibrinogen with semen for 18 hours abolished clot formation on adding the other principle clotting agents. Neither thromboplastin nor prothrombin were inactivated by incubation with semen for 18 hours, of the proteins implicated in blood coagulation only fibrinogen was destroyed.

TABLE II
The Effect of Human Semen on the Clotting Constituents of Beef Blood

Test	Pro throm- bin	Fibri- nogen	Throm- boplas- tin	Semen	NaCl, 0.9 per cent	CaCl ₂ , 0.5 per cent	Time of clotting	Remarks
	cc.	cc.	cc.	cc.	cc.	cc.	sec.	
1	0.5	0.5	0.5	—	0.5	0.2	44	Control—solid clot
2	0.5	0.5	0.5	0.5	—	0.2	80	Solid clot
3	—	0.5	0.5	0.5	0.5	0.2	—	No clot
4	0.5	—	0.5	0.5	0.5	0.2	300	Small amount of clot
5	0.5	0.5	—	0.5	0.5	0.2	320	Solid clot
6	—	0.5	—	0.5	1.0	0.2	—	No clot
7	0.5*	0.5	0.5	0.5*	—	0.2	22	Solid clot
8	0.5	0.5*	0.5	0.5*	—	0.2	85	" "
9	0.5	0.5	0.5*	0.5*	—	0.2	90	
10	0.5‡	0.5	0.5	0.5‡	—	0.2	22	Solid clot
11	0.5	0.5‡	0.5	0.5‡	—	0.2	—	No clot
12	0.5	0.5	0.5‡	0.5‡	—	0.2	15	A few fibres of fibrin appeared at 15 sec. Solid clot in 10 min

The constituents designated * were incubated for 40 minutes at 37°C. and the other elements were then added.

The constituents designated ‡ were incubated for 18 hours and the other elements were then added.

Lytic Action of Human Semen on Normal Human Blood—The blood of healthy persons when mixed with certain amounts of human semen or prostatic fluid will still clot but subsequently liquefy. With equal concentrations of blood and semen, lysis occurs in 1 to 5 hours at room temperature, slightly more rapidly at 37°C. and not at all at 4°C. Serial dilutions of semen in saline were mixed with blood, 1 cc., and observed at 18 hours (Table III). In all instances semen, 0.03 cc., induced lysis, in 4 cases semen, 0.01 cc., liquefied the clot. Twenty samples of prostatic fluid were studied in the same way. Lysis occurred in all with prostatic fluid, 0.02 cc., in 18 hours, and in one fluid 0.002 cc. induced lysis.

Human semen did not liquefy beef plasma. Lysis of dog blood and plasma

clots occurred only twice in 14 experiments and then in no less amounts of semen than 0.5 cc and 0.1 cc. Rabbit plasma was resistant to solution.

Lytic Action of Dog Semen on Coagulated Blood of Normal and Diseased Persons—Lysis of dog blood, clotted after mixing with dog semen, occurred regularly with semen 0.1 cc and larger amounts. Solution of clots produced similarly with rabbit, beef, and rat plasmas occurred with dog semen 1 cc, occasionally with 0.5 cc. amounts, never with less than 0.1 cc of dog semen.

The semen of 10 dogs was tested by serial dilution against the blood of 46 normal persons, the semen was mixed with blood, 1 cc, allowed to clot, and the experiment terminated at 18 hours. In 4 instances the blood did not coagulate, due to destruction of fibrinogen before the clotting was effected, since adding beef fibrinogen induced prompt clotting. Where clotting oc-

TABLE III

Lytic Action of Human Semen on Coagulated Blood of Various Species

1 cc. of whole blood + 1 cc. of secretion, diluted secretion, or saline

Incubation 18 hours, 37°C

Nature of blood	Amount of semen in test					
	None	1 cc.	0.5 cc	0.1 cc	0.05 cc.	0.03 cc.
Normal human	0	L	L	L	L	L
Dog	0	L	I	0	0	0
Rabbit*	0	L	0	0	0	0
Beef*	0	0	0	0	0	0

L, complete lysis, I, incomplete lysis, 0, no lysis

* Citrated plasma, recalcified with CaCl_2 , 1.5 per cent, 0.30 cc

curred, the minimum amount of dog semen causing lysis was 0.016 cc (Table IV). In all cases dog semen, 0.1 cc lysed blood 1 cc, but smaller amounts were often ineffective.

In a similar manner, serial dilutions of dog prostatic fluid were tested for lytic activity against the blood of 24 patients in hospital. Most of the patients had disease of the urinary tract, 7 of them had benign or malignant tumors of the prostate gland. Clotting occurred in all of the tests. In 2 cases, both men with prostatic cancer, no lysis occurred in 18 hours with prostatic fluid, 1 cc. (Table V). In three patients with febrile illness, lysis occurred with prostatic fluid, 1 cc., but not with amounts of 0.5 cc or less. The blood of 19 patients underwent lysis in a normal manner.

General Properties of Fibrinolysis by Semen—When normal blood, 1 cc, was mixed with saline, 1 cc., allowed to clot, and observed 18 hours later, syneresis was always observed, and in the serum a small number (perhaps 1 per cent of the total) of erythrocytes were found lying at the bottom of the tube, detached

TABLE IV

Lytic Action of Dog Semen on Coagulated Blood of Various Species

1 cc. of whole blood + 1 cc. of secretion, diluted secretion or saline.

Incubation 18 hours, 37°C.

Nature of blood	Amount of prostatic secretion in test					
	None	1 cc.	0.5 cc.	0.1 cc.	0.05 cc.	0.033 cc.
Human	0	L	L	L	I	0
Dog	0	L	L	L	I	0
Guinea pig	0	L	L	I	0	0
Rat*	0	L	L	0	0	0
Rabbit*	0	L	L	I	0	0
Beef*	0	L	I	0	0	0

L, complete lysis I incomplete lysis 0 no lysis.

* Citrated plasma, recalcified with CaCl₂, 1.5 per cent, 0.33 cc.

TABLE V

The Lytic Action of Semen of the Dog on Human Blood Permitted to Coagulate

Total volume in test 2 cc. 1 cc. of whole blood + 1 cc. of prostatic fluid, undiluted or diluted with saline

Source	Amount of dog semen in test					Remarks
	1.0 cc.	0.5 cc.	0.1 cc.	0.05 cc.	0.03 cc.	
Healthy person (1)	L	L	0	0	0	
" " (2)	L	L	L	L	0	
" (3)	L	L	L	L	L	
" (4)	L	L	L	0	0	
" (5)	L	L	L	0	0	
" (6)	L	L	L	0	0	
" (7)	L	L	L	I	0	
Patient P M	0	0	0	0	0	Carcinoma of prostate febrile
" A.Y.	0	0	0	0	0	" " afebrile
" A.A.	L	L	L	L	0	" " "
" F.D.	L	I	0	0	0	" " "
" I.K.	L	0	0	0	0	Benign prostatic hypertrophy febrile
" A.H.	L	0	0	0	0	" " "
" J.B.	L	L	L	0	0	" " "
" A.W.	L	0	0	0	0	Tuberculosis of kidney uremia, "

L, lysis I, partial lysis 0 no lysis.

from the clot. It is not known whether this erythrocyte detachment is due to a fibrinolysis. Syneresis is always more pronounced in the presence of erythrocytes than with plasma under the same circumstances for then it is unusual to find any clot retraction.

Fibrinolysis of whole blood by seminal fluids occurs in the absence of bacteria and of spermatozoa, the fluid component of fresh semen, free from evidence of infection and passed through a Seitz filter soon after collection, remained sterile and retained its ability to lyse blood. All samples of semen and prostatic fluids contained the enzyme. The blood of the donor of prostatic fluid was always susceptible to fibrinolysis by his fluid. After fibrinolysis the erythrocytes are intact and appear normal and the specific blood grouping is not removed.

Maintenance of seminal fluids at 60°C for 5 minutes did not interfere with fibrinolytic action, but heating for the same period at 70°C destroyed lytic ability. Prostatic fluid dried in air and stored for 3 weeks retained its lytic property when dissolved in quantity of saline equal to the original volume, the dried powder heated for 2 hours at 105°C was still active. Neither toluol, sulfathiazole crystals in excess, nor merthiolate (sodium ethyl mercuri-thio-salicylate) inhibited fibrinolysis. Inhibitors of oxidation, NaF or KCN in 0.025 molar concentration, did not interfere with fibrinolysis.

The sterile supernatant fluid following fibrinolysis by semen retained its lytic activity when added to fresh blood and allowed to clot. Four such progressive transfers were made without loss of the lytic fraction.

Lytic Action of Human Body Fluids—Fibrinolysis is not a widespread property of body fluids. As control for the lytic effect found in prostatic fluid, several human fluids were examined in a sterile state. These included, urine (10 specimens), saliva passed through a Seitz filter, 21 specimens, gall bladder bile, 6 specimens, hydrocele fluids, 2 lots, 2 spermatocele fluids, 6 normal cerebrospinal fluids, and the secretion of the seminal vesicle from three men. No lysis occurred in any.

Fibrinogenase—When citrated plasma is recalcified, the clear plasma becomes opaque as clotting proceeds. This change in light transmission has been adapted to quantitative study of blood coagulation as a nephelometric procedure by Kugelmass (12) and especially by Nygaard (13) who devised a special apparatus for recording the changes. We investigated the opacification occurring in progressive dilutions of citrated plasma in a photoelectric colorimeter at 37°C with plasma concentrations between 1 and 10 cc (Fig. 1). An approximately linear relationship developed when units of density after clotting are plotted arithmetically against the quantities of fibrin present.

Human citrated blood plasma incubated at 37°C with prostatic fluid of the dog, for short periods of time, shows progressive decrease of opacity after recalcification. In a typical experiment, 5 cc of citrated plasma were incubated with 2 cc of prostatic fluid and at intervals of 6 minutes, 5 cc of saline, and 1.6 cc of calcium chloride were added, in the control tubes prostatic fluid was replaced by saline since the principle electrolytes of dog prostatic fluid are sodium and chloride in 160 milliequivalent concentration per liter (4).

The density resulting from clot formation became progressively less with increasing incubation (Fig 2). After incubation of 30 minutes, neither clotting nor change of density took place. The failure of clot formation was due to destruction of fibrinogen by dog prostatic fluid. However, no decrease of density or of clotting capacity occurred when human semen was added under comparable conditions to the citrated plasma. Indeed, after incubation of human semen with plasma for 4 hours only a slight decrease of density was

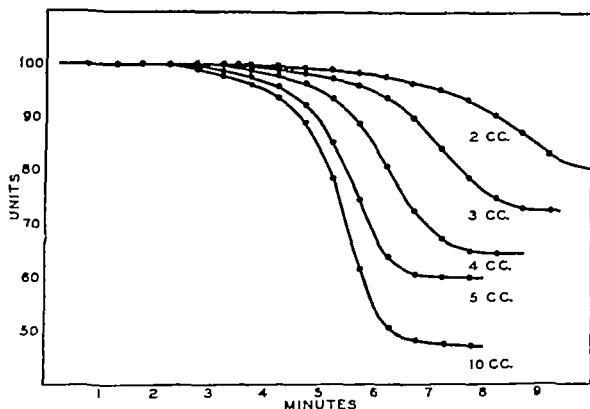


FIG 1 A photoelectric study of the increased density occurring as clot forms in citrated plasma after recalcification. Progressive increases of density occur with increased content of plasma (cubic centimeters) the total volume of fluid in each tube being the same. Ordinates units of galvanometric deflection. Abscissae time in minutes after recalcification.

observed (Fig 3). Human semen possesses only slight power of destroying fibrinogen.

Incubation of citrated plasma, 1 cc., with dog prostatic fluid, 1 cc., for 30 minutes abolished clotting on adding CaCl_2 ; prompt clotting occurred when beef fibrinogen, 0.5 cc., was subsequently added.

The differences in activity of canine and human semen on fibrinogen and fibrin were magnified by dilution of these fluids. Two series of tubes were arranged, all of which contained citrated human plasma, 1 cc., and either human or dog semen, 0.5 cc., in series A, clotting was induced immediately, by adding calcium chloride, 0.25 per cent, 0.25 cc. in series B, calcium chloride

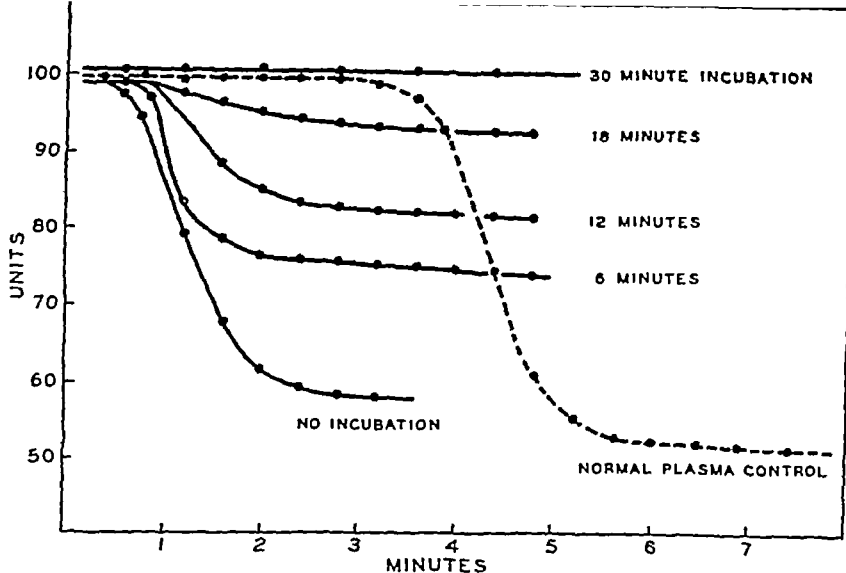


FIG 2 Photoelectric study of the effects of incubation of dog semen with human citrated plasma on clot formation and density occurring after recalcification. A progressive decrease of clot density occurs with increased periods of incubation until contact for 30 minutes results in failure of clotting to take place. When clotting occurs, it begins in all of the tubes containing semen earlier than in the control tubes of plasma without semen, due to the thromboplastic activity of the secretion. Ordinates units of galvanometric deflection. Abscissae time in minutes after recalcification.

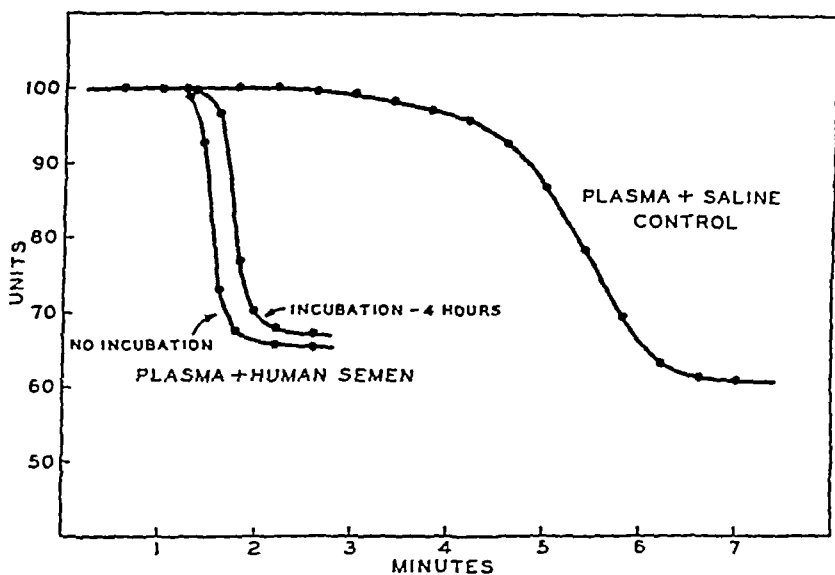


FIG 3 A photoelectric study of the effect of incubation of human semen with citrated plasma on clot formation and density occurring after recalcification. In contrast to dog semen, only a slight decrease of density occurs after 4 hours incubation, the thromboplastic effects are evident as reflected in the increased rate of clotting over that of the control tube of plasma without semen. Ordinates units of galvanometric deflections. Abscissae time in minutes after recalcification.

was added after 18 hours' incubation. At 18 hours in series A, clots containing dog semen, 0.1 cc., were only slightly liquefied, and those containing 0.012 cc. were solid, while accompanying clots containing these amounts of human semen were liquefied (Table VI). At 18 hours in series B, clot could not be induced by recalcification of any of the tubes containing dog semen but the addition of calcium chloride to tubes containing less human semen than 0.1 cc. promptly formed clots which subsequently underwent lysis in

TABLE VI

The Effect of Dog and of Human Semen on Lysis of Fibrin and Fibrinogen

1 cc. of plasma + 0.5 cc. of diluted semen

Series A 0.25 cc. of CaCl_2 added immediately

Series B incubation of fluids for 18 hours before adding CaCl_2

Series A Enzymatic action on clotted plasma			Series B Enzymatic action on liquid plasma		
Content of semen	Coagulation time	Extent of lysis after incubation for 18 hrs.	Coagulation time	Condition of plasma after adding CaCl_2	Time of lysis subsequent to adding CaCl_2
cc.	mins		mins		hrs
Control—no semen	3½	None	4	Solid	No lysis
Dog semen					
0.1	3½	+	Did not coagulate	Liquid	
0.0125	3½	None	"		
Human semen					
0.1	3	++++	Did not coagulate	Liquid	
0.05	3	++++	4	Solid	2
0.025	3	++++	3		4½
0.0125	4	++++	4		No lysis

2 to 5 hours. Further, adding fibrinogen, 0.5 cc., to all tubes in series B which had not clotted after recalcification produced prompt coagulation.

Both human and dog prostatic fluids contain entities capable of destroying fibrin (fibrinolysin) and fibrinogen (fibrinogenase) but in different concentrations. Human semen contains much fibrinolysin, little fibrinogenase, dog semen contains little fibrinolysin, much fibrinogenase.

Thrombin in Semen—The semen of certain dogs has the capacity of clotting fibrinogen, free from prothrombin, and of producing spontaneous clots in oxalated or citrated beef and rabbit plasmas. The calcium content of dog semen is about 0.3 millimols per liter (4). The thrombin activity is weak and requires 5 to 60 minutes before clot is evident. Citrated human plasma is not clotted by dog semen, obviously because human fibrinogen is destroyed by

fibrinogenase before the clot can be formed Human semen does not contain thrombin

To 0.2 cc. of rabbit oxalated plasma diluted with 0.8 cc. of saline, 0.5 cc. of dog semen was added and the tubes maintained at 37°C, spontaneous clotting was observed in 18 minutes. A similar experiment using human plasma instead of rabbit plasma resulted in absence of clot at 10 minutes, the addition of 0.25 cc. of CaCl_2 , 0.25 per cent did not induce clotting. Likewise, replacing dog semen with human semen, 0.5 cc., did not induce clotting in rabbit or human plasma.

To 1.0 cc. of beef fibrinogen, 0.5 cc. of dog prostatic fluid was added, clotting occurred in 20 minutes, replacing dog prostatic fluid with human semen did not result in clotting in 5 hours.

Trypsin in Semen—The semen of both dog and man produced no visible change on coagulated egg albumin in Mett tubes when incubated overnight at 37°C. Using the method of Anson (8) small amounts of a proteolytic enzyme active at pH 7.5, trypsin, were demonstrated in 10 prostatic fluids of dogs, prostatic fluid, 1 cc. liberated chromogen equivalent to 0.029 to 0.15 mg. of tyrosin in 15 minutes. Five human semens, in 1 cc. volume liberated from 0.03 to 0.09 mg. of tyrosine, in 15 minutes, two human semens did not contain trypsin.

DISCUSSION

The mechanism by which human semen coagulates is not clear, and the problem is complicated by the difficulty of securing semen prior to clotting. The finding of fibrinogen and thromboplastin is evidence that seminal clotting resembles blood clotting, however, thrombin and prothrombin were not demonstrated after liquefaction. The abundance of citrate, which is sufficient to bind all of the calcium ions in many semens, provides an obstacle to the inference that the clotting of blood and semen is due to an identical mechanism. The presence of an active fibrinolytic agent would readily explain liquefaction, if, as seems likely, fibrin were the cause of the seminal clot.

The fibrinolysin in semen is derived from the prostate gland, and it was present in large amount in all of the prostatic fluids examined. This constant occurrence establishes a new function for the prostate. We have been unable to find previous observations relative to the action of genital secretions on fibrin except by Kurczok and Miller (14), who were unable to demonstrate an effect of semen on fibrin, the source of which was unspecified. While all of the semens became liquid spontaneously in less than 10 minutes in our tests, the most rapid liquefaction of blood, 1 cc., by semen, 1 cc., occurred in 30 minutes. The lysis of blood clot by semen occurs less rapidly than lysis of the clotted semen itself.

Fibrinolysis without implication of the agent involved has been studied by several workers since its discovery in serum by Dastre (15). Nolf (16) re-

garded a slow aseptic fibrinolysis to be a natural sequel of plasma coagulation. Judine (17) observed that the blood of men in profound traumatic shock did not coagulate, further, that the blood of healthy persons, meeting a violent death, coagulated rapidly but within several hours became liquid and did not thereafter coagulate. Macfarlane (18) found that frequently the clotted blood of patients after surgical operations subsequently underwent complete lysis under aseptic conditions within 24 hours.

The effect of "chloroform semen" is germane to the present discussion. Howell (19) and Minot (20) discovered that clotting occurred in oxalated plasma to which chloroform had been added and Nolf (21) observed that the serum obtained from such clots is fibrinolytic. These findings have been confirmed by Tagnon (22, 23) who obtained from such serum, from which the chloroform had been removed, a globulin with marked fibrinolytic properties the addition of this globulin to fibrinogen produced no clot but effected complete lysis of fibrinogen, while in the presence of prothrombin a clot formed which sometimes underwent fibrinolysis. The action on blood plasma and fibrinogen of this globulin resembles the effect of trypsin as described by Eagle and Harris (24). Ferguson and Erickson (25) in studying the clotting action of crystalline trypsin on citrated plasma, observed that clots so obtained undergo fibrinolysis within a few minutes they found that trypsin, in 1 to 2 mg. amounts was optimal for the clotting of 1 cc. of citrated dog plasma.

Many points of similarity occur between the fibrinolysin of semen and that discovered by Tillett and Garner (26) in hemolytic streptococci. Both agents lyse normal human blood clot easily but act only after prolonged periods of time and in high concentration, when at all, on rabbit blood. In disease, at times, the blood of certain patients is totally resistant to lysis by semen, Tillett, Edwards, and Garner (27) got the same result with the fibrinolysin from streptococci in their patients with streptococcal infections. Further the active principle is demonstrable in dissolved fibrin even after incubation for 18 hours in both cases. Slight differences occur however. Garner and Tillett (28) found that streptococcal fibrinolysin resisted heating at 100°C for 60 minutes, while we observed that semen is inactivated by heating to 70°C for 5 minutes. Seminal fibrinolysin retains its activity for months in the refrigerator while that from streptococci deteriorates in several weeks.

In relating these observations to the effect of semen in the lysis of fibrin and fibrinogen, it should be stated that all of the samples of dog semen, and some human semens contained small amounts of trypsin, other human semens were free from trypsin. All samples were inactive in destroying beef fibrinogen and fibrin.

Both human and dog semens contain factors capable of inactivating fibrin and fibrinogen but in different and inverse proportions, human fluid contained fibrinolysin with greater activity than dog semen, while dog semen exerted

far greater fibrinogenase activity than human semen. It was readily possible by dilution to eliminate the weaker activity and to retain the stronger, this is evidence for the presence of two distinct proteolytic agents acting on fibrin and fibrinogen respectively. Garner and Tillett (29) observed that "solutions of human fibrinogen after brief incubation with fibrinolysin lose the capacity to form thrombin." The observed quantitative and species differences of proteolysis in semens do not fall in with the interpretation of these workers, whose observation on streptococcal filtrate with respect to fibrinogen is the same as ours on dog semen. We conclude that the agent in semen which liquefies fibrin resembles closely or is identical with the fibrinolysin of Tillett and Garner, and that the inactivation of fibrinogen is due to a separate agent, fibrinogenase.

SUMMARY

Certain specimens of human semen shorten the coagulation time of whole blood because of the presence of active thromboplastic agents, while other samples prolong its coagulation time. Human prostatic fluid in large amounts always delays or abolishes blood coagulation. The delay or absence of clotting is counteracted by adding calcium ions and is due to the large concentration of citrate in prostatic fluid and in some semens.

While most specimens of dog semen shorten the coagulation time of blood because of their thromboplastic activity, certain specimens render blood incoagulable or delay coagulation, in contrast to human semen, this adverse effect on coagulation is not overcome with calcium ions and is due to a different mechanism, the lysis of fibrinogen. The citrate content of dog prostatic fluid is small.

Human semen which has become liquefied does not contain thrombin or prothrombin, but fibrinogen and thromboplastic substances are present. Beef fibrinogen added to semen is destroyed by incubation for 18 hours, but added prothrombin and thromboplastic substances are still present after this treatment. Dog semen, in some instances, contains small amounts of thrombin.

The semens of man and dog contain a fibrinolysin for human blood which seems not to differ greatly from the fibrinolysin associated with hemolytic streptococci. The blood of the donor of prostatic fluid is susceptible to fibrinolysis by this fluid. However, the blood of persons with some diseases, is absolutely resistant to the action of seminal fibrinolysin. In how many diseases this happens has not yet been determined.

The semens of man and dog both contain an agent capable of inactivating fibrinogen, but in different amounts. This activity may be called fibrinogenase. Human semen is rich in fibrinolysin, poor in fibrinogenase, dog semen is rich in fibrinogenase, poor in fibrinolysin. These species differences, together with the fact that it is easy by appropriate dilution to retain the stronger proteolytic

agent and eliminate the weaker one, imply that fibrinolysin and fibrinogenase are different entities

Dog semen, and less constantly human semen, contain very small amounts of trypsin.

All of these proteolytic agents derive from the prostate gland, their secretion in prostatic fluid constitutes a hitherto undescribed function for the prostate gland.

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A NATURAL ANTIBODY THAT REACTS IN VITRO WITH A SEDIMENTABLE CONSTITUENT OF NORMAL TISSUE CELLS*

I. DEMONSTRATION OF THE PHENOMENON

By JOHN G KIDD M D, AND WILLIAM F FRIEDEWALD, M.D

(From the Laboratories of The Rockefeller Institute for Medical Research)

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While making serological studies of transplanted rabbit cancers by the methods that disclosed a distinctive substance in the Brown-Pearce carcinoma (1), the blood serum of normal rabbits has been found to fix complement when mixed with fresh saline extracts of normal rabbit tissues, and control tests have shown that anticomplementary effects are not responsible for the reaction. The phenomenon appears to be due, as will now be shown, to a natural antibody that reacts *in vitro* with a sedimentable constituent of many normal tissue cells.

Methods and Materials

The natural antibody and the substance with which it reacts were studied by means of a standardized complement fixation test in which 2 units of complement were employed with 2 hours at room temperature for fixation. The procedure was in most respects the same as used in previous studies from this laboratory (1), but differed in the method whereby the test antigens were prepared. Fresh or frozen tissues instead of glycerolated ones were employed for this purpose, and the antigen-extracts were not heated at 56°C as in the preceding work,—variations in method which proved significant, as will become evident further on.

The test antigens were made from normal tissues (liver kidney brain etc.), which had been procured with aseptic precautions these were used fresh or after preservation in the frozen state (−22°C.) for periods up to several months. They were ground with sand in a mortar and the ground paste suspended in physiological saline (1 10 to 1 40 or more) and spun for 20 minutes at 4400 R.P.M. in the 51° angle-head International centrifuge. The unheated supernatant liquids, slightly to moderately opalescent but free from gross particles proved notably effective in the complement fixation tests, especially when used the same day.

Normal sera were procured by bleeding rabbits either from an ear vein or from the heart, allowing the blood to clot in paraffined tubes and subsequently clearing in the

* Preliminary note in the *Proceedings of the Society for Experimental Biology and Medicine*, 1941, 47, 128

centrifuge The specimens were stored in stoppered tubes in the refrigerator at about 4°C Many of them were tested repeatedly during periods of several months without perceptible change in effectiveness and without becoming anticomplementary All were heated at 56°C for 30 minutes immediately prior to use, to inactivate complement

Complement was got by bleeding 10 to 20 guinea pigs As soon as the serum could be collected and pooled, it was distributed in small vials and dried from the frozen state in the Flosdorf-Mudd apparatus Immediately before each experiment, the dried serum was dissolved in physiological saline and titrated with the actual hemolytic system to be employed Two full units of complement were invariably used

The *hemolytic system* consisted of rabbit serum containing immune sheep hemolysin in high titer, and thrice washed sheep erythrocytes The hemolysin was titrated at infrequent intervals, it was diluted so that 0.2 cc. contained 4 hemolytic units The 5 per cent washed sheep cells were sensitized with an equal volume of the diluted hemolysin, the mixture standing 10 minutes at 37°C immediately before use in the test

The serum, complement, and test antigens were mixed so that complement was present when the other two reagents came together The mixtures, containing 0.2 cc of each reagent, were allowed to stand 2 hours at room temperature, then 0.4 cc. of sensitized erythrocytes was added Readings were made after 30 minutes at 37°C and again after the tubes had stood overnight in the refrigerator The final readings, which seldom differed significantly from the first ones, are recorded in the published tables in terms of fixation ++++ = complete fixation (no hemolysis), +++ = about 75 per cent fixation, ++ = about 50 per cent fixation, + = about 25 per cent fixation, 0 = no fixation (complete hemolysis)

Control tests were made in every experiment for *anticomplementary effects*, using double volumes of each dilution of serum and of test antigen In general the sera were practically never anticomplementary in dilutions of 1:4 or higher and usually not at all or only very slightly so at 1:2, the largest amount utilized The test antigens occasionally manifested slight or moderate anticomplementary effects in dilutions of 1:10, but practically never in dilutions of 1:20 and never in our experience at 1:40 Neither the sera nor the antigens were anticomplementary in any of the experiments recorded in the present paper

Incidence and Characteristics of the Naturally Occurring Serum Principle

The results of many experiments have made plain the fact that the blood serum of nearly all normal adult rabbits will regularly fix complement in greater or less degree in mixture with unheated saline extracts of fresh or frozen normal rabbit tissues, whereas the sera of very young rabbits have always failed to do so Table I gives the results of an experiment that can be considered typical The sera of 10 normal adult rabbits all fixed complement in mixture with a freshly prepared 1:20 saline extract of normal rabbit liver, some in dilutions as high as 1:32, while specimens from 13 rabbits up to 24 days old of the same hybrid sort, from 7 litters, all failed to react with the test antigen The positive reactions could not have been due to a summation of

anticomplementary effects, for the sera were not anticomplementary when tested concurrently, even in control tubes containing 0.4 cc. of the 1:2 dilutions, nor was the antigen anticomplementary in control tests containing two

TABLE I
Tests for the Serum Principle in the Blood of Adult and Very Young Rabbits

Source of serum	Rabbit No	Age	Complement fixation tests					
			Serum dilution					
			1:2	1:4	1:8	1:16	1:32	1:64
		<i>days</i>						
Normal gray-brown domestic rabbits weighing 2 kg. or more	15-84		++++	++++	++++	++++	+++±	0
	15-87		+++±	++++	++++	+++±	+	0
	15-88		++++	++++	++++	++++	++++	±
	15-90		++++	++++	++++	++++	+	0
	15-98		++++	++++	++++	++++	0	0
	15-99		++++	++++	+++±	±	0	0
	16-00		++++	++++	++++	+++	0	0
	16-01		++++	++++	+++±	+±	0	0
	16-02		+++±	++++	++++	++	0	0
	16-03		++++	++++	++++	+++±	0	0
Normal gray brown domestic rabbits less than 4 wks. old	1A*	24	0	0	0	0	0	0
	2B	18	0	0	0	0	0	0
	3C	19	0	0	0	0	0	0
	4D	20	0	0	0	0	0	0
	5D	"	0	0	0	0	0	0
	6D	"	0	0	0	0	0	0
	7E		0	0	0	0	0	0
	8E		0	0	0	0	0	0
	9F	16	0	0	0	0	0	0
	10F		0	0	0	0	0	0
	11F		0	0	0	0	0	0
	12G	"	0	0	0	0	0	0
	13G		0	0	0	0	0	0

Antigen, 1:20 saline extract of fresh normal rabbit liver (D.R. 5-73)

2 units of complement in all tubes, as also in all of the tables that follow

++++ = complete fixation 0 = no fixation.

None of the sera was anticomplementary when tested concurrently in double volume, nor was the antigen. The same holds true in all of the tables to follow

* A B C, etc. = various litters.

and four times the amount used in the recorded experiment. This holds true also for the experiments that follow

Like the generality of antibodies, the naturally occurring serum principle can be precipitated with ammonium sulfate (Table II). Yet it is inactivated when sera containing it are heated at 65°C for 30 minutes (Table III)—a

procedure that has no noteworthy effect upon the generality of antibodies in rabbit blood, as is well known (2)

TABLE II
Precipitation of the Serum Principle with Ammonium Sulfate

Source of serum	Fraction	Serum dilution				
		1 4	1 8	1 16	1 32	1 64
Normal rabbit 16-23	Whole serum	++++	++++	++++	++++	+++
	Globulin fraction*	++++	++++	++++	++++	+++
Normal rabbit 16-28	Whole serum	++++	++++	++++	++++	±
	Globulin fraction*	++++	++++	++++	++++	±

* Precipitated with half saturated ammonium sulfate and dialyzed against 0.9 per cent NaCl.

Antigen, 1:40 saline extract of frozen normal rabbit liver (D.R. 3:72)

TABLE III
Incidence, Titer, and Heat Lability of the Serum Principle

Normal rabbit sera	Complement fixation tests									
	Sera heated at 56 C for 30 min					Sera heated at 65 C for 30 min				
	Serum dilution					Serum dilution				
	1 2	1 4	1 8	1 16	1 32	1 2	1 4	1 8	1 16	1 32
D.R. 5-65	++++	++++	++++	++++	++++	0	0	0	0	0
5-69	++++	++++	++++	++++	++++	0	0	0	0	0
5-70	++++	++++	++++	++++	++++	0	0	0	0	0
5-67	++++±	++++	++++	++++	++++	0	0	0	0	0
5-75	++++	++++	++++	++++	++++	0	0	0	0	0
5-71	+++	++++	++++	++++	++++±	0	0	0	0	0
5-74	++++	++++	++++	++++±	±	0	0	0	0	0
5-73	++++	+++	++±	0	0	0	0	0	0	0
5-68	++++	+++	++	0	0	0	0	0	0	0
5-66	+++	+++	+++	0	0	0	0	0	0	0
5-72	+++	++	+	0	0	0	0	0	0	0
5-76	+++±	±±	0	0	0	0	0	0	0	0

Antigen, 1:40 saline extract of frozen normal rabbit liver (D.R. 3:72)

D.R. = domestic rabbit.

Since the fact has long been recognized that other naturally occurring serum principles, generally termed natural antibodies, are absent from the blood of newly born individuals and that they are more labile to heat than induced ones (3), it seemed possible that the serum principle now under study might also prove to be a natural antibody. Hence it was compared as to titer and to heat lability with two natural antibodies known to be present in the serum

of normal adult rabbits, viz., natural sheep hemolysin and natural Wassermann reagin

The sera of 6 adult gray brown domestic rabbits were employed, which were known from the experiment of Table III to contain various amounts of the serum principle under study. Specimens were heated at 56°C. and 65°C. and tested in various dilutions for capacity to hemolyze 5 per cent washed sheep cells in the presence of 4 units of complement (natural sheep hemolysin) for capacity to fix complement in mixture with a 1 per cent cholesterolized Wassermann antigen¹ (natural Wassermann reagin), and for capacity to fix complement in mixture with a 1:20 saline extract of frozen normal rabbit liver (serum principle under study)

Table IV shows the results of the comparative tests. It will be seen that in general the titers of the three serum constituents varied together. The sera of rabbits 5-69, 5-75, and 5-65, for example, reacted notably well in all three tests, while the specimens of 5-72 and 5-76 did poorly. Serum 5-70 provides an exception in that it had little natural power to hemolyze sheep cells but much to react with the Wassermann and normal rabbit tissue antigens. (For other such exceptions see Table V further on.) Heating the sera at 65°C. rendered them completely ineffective, or nearly so, in all of the tests. It will be noted, however, that the serum specimen of rabbit 5-65 was exceptional in that it still retained a small proportion of its capacity to hemolyze sheep cells, and so too in slighter degree did those of rabbits 5-76 and 5-75.²

An experiment was next undertaken to find whether serum principles of the three types can be selectively absorbed.

Sera from 5 normal adult agouti rabbits were employed. 10 cc. volumes of each were mixed with 30 cc. of 50 per cent washed sheep cells, 30 cc. of 1 per cent cholesterolized Wassermann antigen, 30 cc. of 1:10 saline extract of normal rabbit liver and 30 cc. of physiological saline, respectively. All of the mixtures were kept 2 hours at 37°C. then put overnight in the refrigerator. The ones containing sheep cells were next spun at low speed in the ordinary centrifuge and the others at 25 000 R.P.M. for 1 hour in the air-driven centrifuge—an amount of centrifugation that will not perceptibly throw down other rabbit antibodies, as has been repeatedly observed (4).

The absorbed and unabsorbed supernatant liquids were then tested as in the preceding experiment. Table V shows the results. Absorption with the sheep erythrocytes had removed completely the capacity of all of the sera to hemolyze sheep cells without affecting their ability to fix complement in mixture with the Wassermann

¹ Supplied by the Diagnostic Laboratories of the New York City Board of Health through the generosity of Dr. G. I. Steffen.

² The exceptions are important as indicating the fact that 65°C. for 20 to 30 minutes does not always completely inactivate the natural antibodies of normal rabbit's blood. On several occasions sera with unusually high titers of the natural tissue antibody have retained slight activity when thus heated.

TABLE IV
Titer and Heat Lability of Various Natural Antibodies in Normal Rabbit Serum

Normal rabbit sera	Tests for																			
	Natural sheep hemolysin*										Natural Wassermann reagin†									
	Serum dilution					Serum dilution					Serum dilution					Serum dilution				
Heated (30 min)	1 2	1 4	1 8	1 16	1 32	1 64	1 2	1 4	1 8	1 16	1 32	1 64	1 2	1 4	1 8	1 16	1 32	1 64	1 2	1 4
56°C	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
65°C	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+

See text for procedures of the three types of tests.

* Readings in terms of hemolysis

† Readings in terms of fixation

TABLE V
Selective Absorption of Various Natural Antibodies from Normal Rabbit Sera

Normal rabbit sera	Absorbed with	Tests for															
		Natural sheep hemolysin								Natural Wassermann reagin†							
		Serum dilution								Serum dilution							
		1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	
16-00	NTU, saline control	+++	+++	+++	+++	±	+++	+++	+++	±	0	+++	+++	0	0	1:64	0
15-88	" "	+++	+++	+++	+++	+++	+++	+++	+++	±	0	+++	+++	±	±	0	0
15-84	" "	+++	+++	+++	+++	+	+++	+++	+++	+++	0	+++	+++	+++	+	0	0
5-75	" "	+++	+++	+++	+++	±	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
5-65	" "	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
16-00	Sheep erythrocytes	0	0	0	0	0	+++	+++	+++	+	0	+++	+++	0	0	0	0
15-88	" "	0	0	0	0	0	+++	+++	+++	+++	0	+++	+++	+++	+	0	0
15-84	" "	0	0	0	0	0	+++	+++	+++	+++	0	+++	+++	+++	±	0	0
5-75	" "	0	0	0	0	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
5-65	" "	0	0	0	0	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
16-00	Wassermann antigen	+++	+++	+++	+++	±	+++	+++	+++	0	0	+++	+++	0	0	0	0
15-88	" "	+++	+++	+++	+++	+++	+++	0	0	0	0	+++	+++	+++	+	0	0
15-84	" "	+++	+++	+++	+++	+	+++	0	0	0	0	+++	+++	+++	±	0	0
5-75	" "	+++	+++	+++	+++	±	+++	0	0	0	0	+++	+++	+++	+++	±	±
5-65	" "	+++	+++	+++	+++	+++	+++	0	0	0	0	+++	+++	+++	+++	+++	+++
16-00	Rabbit liver extract	+++	+++	+++	+++	±	+++	+++	+++	0	0	0	0	0	0	0	0
15-88	" "	+++	+++	+++	+++	±	+++	+++	+++	0	0	0	0	0	0	0	0
15-84	" "	+++	+++	+++	+++	±	+++	+++	+++	0	0	0	0	0	0	0	0
5-75	" "	+++	+++	+++	+++	±	+++	+++	+++	+++	+++	+++	+++	+++	+++	0	0
5-65	" "	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0	0

* Readings in terms of hemolysis.

† Readings in terms of fixation

or the normal rabbit tissue substances. Likewise, the Wassermann substance absorbed the natural reagin completely without affecting the other two serum constituents. The extract of normal rabbit liver absorbed almost completely the capacity of the various sera to react with a similar extract, and it reduced their ability to fix complement in mixture with the Wassermann substance, but it had no effect upon the natural hemolysin.

In general the titer of the serum principle under study ran parallel to that of the two natural antibodies with which it was compared, though there were

TABLE VI
Reaction of the Natural Antibody with Saline Extracts of Normal Rabbit Tissues

Source of antigen		Antigen dilution					
Rabbit	Organ	1 20	1 40	1 80	1 160	1 320	1 640
5 69	Kidney	++++	++++	++++	++++	++++	+++
	Liver	++++	++++	++++	++++	+++±	±±
	Lung	++++	++++	++++	+++±	0	0
	Brain	++++	++++	++++	±	0	0
	Spleen	++++	++++	+++	0	0	0
	Heart	++++	++±	0	0	0	0
	Muscle	±±	0	0	0	0	0
5-76	Kidney	++++	++++	++++	++++	++++	++
	Liver	++++	++++	++++	++++	+++±	0
	Lung	++++	++++	+++±	0	0	0
	Brain	++++	++++	+++±	±	0	0
	Spleen	++++	++++	±±	0	0	0
	Heart	+++±	0	0	0	0	0
	Muscle	0	0	0	0	0	0

Antigens, saline extracts of frozen normal tissues as indicated.

Normal rabbit serum D R 5-65, 1 12,—known from previous tests to contain the natural tissue antibody (see Table III)

noteworthy exceptions (Tables IV and V). The results of absorption tests, however, proved that the new serum principle has affinities wholly distinct from those of the natural antibodies mentioned (Table V). Since its incidence and heat lability proved similar to those of the so called natural antibodies, and because it possesses a strict affinity for a sedimentable constituent of normal tissue cells (about which more further on), it has been termed the natural tissue antibody. More will be said about it in the discussion, after scrutiny of the tissue substance with which it reacts *in vitro*.

Characteristics of the Tissue Substance with Which the Natural Antibody Reacts

Normal rabbit livers provided the antigens for the preceding complement fixation tests. Will other rabbit organs serve as well? The experiment sum-

marized in Table VI yields an answer to the question. For saline extracts, made as already described from the kidney, liver, lung, brain, spleen, and heart tissues of two rabbits, all reacted with a serum known to contain the natural tissue antibody, their capacity to do so varying from much to little in the order given. Saline extracts of the voluntary muscles had comparatively

TABLE VII

Reaction of the Natural Antibody with Centrifugalized Extracts of Normal Rabbit Tissues

Source of antigen	Portion tested	Centrifugation (60 min.)	Antigen dilution						
			1:10	1:20	1:40	1:80	1:160	1:320	1:640
Normal rabbit liver— frozen (D.R. 5-73)	Whole extract	D.F.M.	++++	++++	++++	++++	++++	++++	++
	Supernatant	7 500	++++	++++	++++	++++	++++	++	0
		15 000	++++	++++	++	0	0	0	0
		20 000	+	0	0	0	0	0	0
		25 000	0	0	0	0	0	0	0
		30 000	0	0	0	0	0	0	0
	Resuspended sediment	7 500	++++	++++	++++	++++	++++	+++	0
		15 000	++++	++++	++++	++++	++++	++++	++
		20 000	++++	++++	++++	++++	++++	++++	++
		25 000	++++	++++	++++	++++	++++	++++	++
		30 000	++++	++++	++++	++++	++++	++++	++
Normal rabbit kidney —fresh (D.R. 4-64)	Whole extract		++++	++++	++++	++++	++++	++++	++++
	Supernatant	7 500	++++	++++	++++	++++	++++	0	0
		15 000	++++	++++	+++	±	0	0	0
		20 000	++++	±	0	0	0	0	0
		25 000	±	0	0	0	0	0	0
		30 000	0	0	0	0	0	0	0
	Resuspended sediment	7 500	++++	++++	++++	++++	++++	+++	0
		15 000	++++	++++	++++	++++	++++	++++	0
		20 000	++++	++++	++++	++++	++++	++++	++
		25 000	++++	++++	++++	++++	++++	++++	++
		30 000	++++	++++	++++	++++	++++	++++	++

Antigens extracted in dilute phosphate buffer (approximately 0.05 M pH 7.3)

Normal rabbit serum D.R. 5-70 1:8, known to contain the natural tissue antibody (see Table III)

little or no such ability. The results have often been confirmed: saline extracts of the organs just mentioned, obtained from many normal rabbits, invariably fixed complement in mixture with normal rabbit sera known to contain the natural antibody, and their ability to do so was in general much as shown in Table VI. Numerous tests failed to disclose the effective substance in either the erythrocytes or the blood serum of normal rabbits, though it was found in noteworthy amounts in extracts of whole rabbit embryos. Experiments will be reported further on which show that it is also present in extracts of the tissues of alien species.

It seemed important to learn whether the material with which the natural antibody reacts can be thrown down readily in the high-speed centrifuge. For this is a property shared alike by many viruses and by certain constituents of normal and neoplastic tissues (5), including the distinctive substance of the Brown-Pearce tumor (1).

To decide about this, 110 extracts were made in dilute phosphate buffer (Sørensen's, approximately 0.005 M, pH 7.3) of the frozen normal liver of rabbit D R (domestic rabbit) 5-73 and of the fresh kidney of D R 4-64. The extracts were spun briefly to throw down the coarse tissue debris, and the densely opalescent supernatant liquids divided into 7 cc. lots. A 3 cc. portion of each material was saved as such (whole extract), and others were spun in the air-driven centrifuge for 1 hour at speeds

Complement Fixation Tests with Sera of (a) Normal and (b) S.

Source of serum	Rabbit No	Alcoholic extracts							
		Normal rabbit liver							
		Antigen dilution						1 25	1 5
		1 25	1 50	1 100	1 200	1 400	1 800		
(a) Normal rabbits	13-94	0	0	0	0	0	0	0	0
	13-95	0	0	0	0	0	0	0	0
	13-92	0	0	0	0	0	0	0	0
	13-93	0	0	0	0	0	0	0	0
(b) Rabbits with experimental syphilis	3-25	++++	++++	+++±	+++±	±	0	++++	+++
	3-17	++++	+++	+	0	0	0	++++	+++

Complement, 2 units in all tubes

Sera diluted 1:4. Heated 56°C

The antigens were made from the tissues of a single normal rabbit—D R 2-72

of 7,500, 15,000, 20,000, and 30,000 R P M., respectively. The supernatant liquids were poured off and the sedimented materials resuspended in the original volume of dilute buffer. The materials were kept cold during the entire procedure, and the sedimented ones were resuspended carefully and uniformly, according to a procedure already described (1).

The results of tests with the various fractions are set down in Table VII. Both of the whole extracts proved notably effective in mixture with a 1:8 dilution of a serum that was known to contain the natural tissue antibody (D R 5-70). The supernatant fluids of the materials spun at 7,500 R P M. fixed complement in several dilutions in the tests, but they did so in lesser degree than did the corresponding resuspended sediments. The supernatant fluids of the materials spun twice as fast were much less effective and the resuspended sediments much more so. Practically all of the effective substance was thrown down in the materials spun at 20, 25, and 30 thousand R P M., comparatively little or none of it remaining in the supernatant liquids.

From Tables VI and VII it becomes clear that the substance with which the natural tissue antibody reacts is widely distributed in the tissues of normal

rabbits and that it can be thrown down readily in the high-speed centrifuge, comparatively little or none of the effective material remaining in the supernatant liquid when extracts containing it are spun at 25,000 R.P.M. (45,400 g) for 1 hour. The implications of the finding will be discussed further on.

Certain of the other properties of the reactive tissue constituent will now be described.

Effect of Alcohol on the Tissue Substance—Table VIII shows the results of an experiment in which the sera of 4 normal adult rabbits were tested in mixture with alcoholic and saline extracts of normal rabbit liver and heart. The sera of 2 rabbits with experimental syphilis were included for comparison. The alcoholic extracts were made by grinding the fresh tissues as usual and extracting overnight in 5 volumes

Mixture with Alcoholic and Saline Extracts of Normal Rabbit Tissues

			Saline extracts									
Sera			Normal rabbit liver					Normal rabbit heart				
			Antigen dilution					Antigen dilution				
	1:400	1:800	1:20	1:40	1:80	1:160	1:320	1:20	1:40	1:80	1:160	1:320
0	0	0	++++	++++	++++	+	0	++++	++++	±	0	0
0	0	0	++++	++++	±	0	0	++++	0	0	0	0
0	0	0	++++	++++	+	±	0	++++	±	0	0	0
0	0	0	++++	±	0	0	0	±	0	0	0	0
+	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++
+	±	0	++++	++++	+	0	0	++++	++++	±	0	0

of 95 per cent alcohol. The filtered alcoholic extracts (1:5) were then added slowly to 4 volumes of saline so as to form suspensions of maximum turbidity (1:25) which were used in the tests as such and after further dilution with saline, in comparison with saline extracts made as usual of the same tissues which had been kept overnight at -22°C .

- None of the 4 normal rabbit sera reacted with the alcoholic extracts, though both of the syphilitic rabbit sera did so (Table VIII). As was expected, all of the sera reacted with the saline extracts of the normal tissues. Manifestly, the tissue substance with which the natural antibody reacts is either destroyed by alcohol or does not come away into it.

Effect of Heat—Table IX shows the results of one of several similar experiments. A 1:10 extract was made in dilute phosphate buffer (pH 7.3) of the frozen normal liver of rabbit 5-69, and likewise a 1:10 extract in 0.9 per cent sodium chloride solution of the frozen normal liver of rabbit 5-73. These were spun at 4400 R.P.M. for 20 minutes and the supernatant liquids, which were opalescent but free from gross particles, were removed. They will be referred to as whole extracts. A portion of each whole extract was spun at 25,000 R.P.M. for an hour and the sediments carefully resuspended

in buffer and saline respectively, to provide suspensions of the active material partially freed from extraneous materials. The two materials were then divided into several lots of 30 cc. One lot of each was kept unheated as controls and the rest were heated for 30 minutes in water baths at temperatures of 56, 60, 65, 70, and 75°C, respectively. Heavy precipitates formed in some of the heated materials (notably

TABLE IX
Effect of Heat on the Reactive Constituent of Normal Rabbit Tissues

Source of test antigen	Heating 30 min	Gross appearance	Complement fixation tests*						
			Antigen dilution						
			1:10	1:20	1:40	1:80	1:160	1:320	1:640
Whole liver extract in dilute buffer pH 7.3 (D.R. 5-69)	Unheated	Opalescent	++++	++++	++++	++++	++++	++++	++++
	56°C	Heavy precipitate	+	++	++	+	0	0	0
	60°	" "	0	0	0	0	0	0	0
	65°	" "	0	0	0	0	0	0	0
	70°	" "	0	0	0	0	0	0	0
	75°	" "	0	0	0	0	0	0	0
Whole liver extract in physiological saline (D.R. 5-73)	Unheated	Opalescent	++++	++++	++++	++++	++++	++++	++++
	56°C	Heavy precipitate	+	++	++	++	++	±	0
	60°	" "	+	+	+	+	+	±	0
	65°	" "	0	0	0	0	0	0	0
	70°	" "	0	0	0	0	0	0	0
	75°	" "	0	0	0	0	0	0	0
Partially purified liver extract in buffer (D.R. 5-69)	Unheated	Slightly opalescent	++++	++++	++++	++++	++++	++++	++++
	56°C	" "	++++	++++	++++	++++	++	0	0
	60°	" "	++++	++++	++++	++++	+	0	0
	65°	Opalescent	++++	++++	++++	+++	0	0	0
	70°	" "	++	++++	+++	±	0	0	0
	75°	" "	++++	++++	+++	±	0	0	0
Partially purified liver extract in physiological saline (D.R. 5-73)	Unheated	Slightly opalescent	++++	++++	++++	++++	++++	+++	0
	56°C	" "	++++	+++	+++	+++	+++	±	0
	60°	" "	++++	+++	++	++	+	0	0
	65°	Moderate precipitate	±	±	±	0	0	0	0
	70°	Heavy " "	+	±	±	0	0	0	0
	75°	" "	+	0	0	0	0	0	0

* Normal rabbit serum D.R. 5-69, 1:8, known to contain the natural tissue antibody in high titer

† Spun down once in the high speed centrifuge (25,000 R.P.M. for 1 hour) and resuspended in the original volume of fluid.

in the whole extracts—see the table) but these broke up readily and were resuspended before use in the complement fixation tests.

From Table IX it is seen that the whole extracts heated at 56°C and 60°C reacted very poorly in the tests and those heated at higher temperatures not at all. The results were different, however, with the partially purified materials. These were not visibly affected by 56°C and 60°C, though the higher temperatures brought about an increase in opalescence of the ones suspended in dilute buffer and moderate and heavy precipitates in the materials suspended in physiological saline. The partially

purified materials heated at 56°C. were less than half as effective as the corresponding unheated ones, and the higher temperatures resulted in a progressive loss in activity. But this was not completely destroyed even at 75°C. It is noteworthy that the material suspended in the dilute phosphate buffer was more effective to begin with than that suspended in physiological saline.

Other experiments yielded similar results. In one, 37°C. and 45°C. for 30 minutes brought about no perceptible change in the activity of materials derived from normal rabbit liver and kidney that had been washed twice in the high-speed centrifuge and resuspended in dilute phosphate buffer. But 56°C. reduced their effectiveness by more than half and the higher temperatures reduced it still more though 75°C. failed to inactivate the materials completely. In still another test, twice washed liver material which had stood 2 weeks in the refrigerator in suspension in dilute buffer proved moderately effective when used unheated but was almost completely devoid of activity after heating for half an hour at 56°C. and at 65°C.

Effects of Various Extracts—Incidental observations revealed that much of the active material comes away from ground tissues into physiological saline and even more into dilute phosphate buffer (Sørensen's, pH 7.3 approximately 0.005 M). Tyrode's solution carefully adjusted to pH 7.3 with CO₂ proved poor as an extractive, less than half as much of the active substance being present in Tyrode-extracts as in 0.9 per cent saline-extracts of identical normal tissues.

Inactivation upon Standing—Standing several days or even overnight in the refrigerator at about 4°C. had a markedly deleterious effect upon saline suspensions containing the active material from kidney or liver. These soon became more opalescent than when fresh, and after a day or two flocculations were visible in them, and the materials were found on test to have lost much or all of their ability to react with sera containing the natural antibody. The deleterious effects were noted also in buffer extracts, but were less marked in these.

Glycerolated and Frozen Tissues as a Source of Antigen—A comparison of glycerolated and frozen tissues proved that the latter were superior as a source of the tissue substance. The liver, kidney, and testicle tissues from 2 normal rabbits diced in pieces a few millimeters across and kept in 50 per cent glycerol Locke's solution for 10 days yielded less than half as much of the active material as did specimens of the same tissues kept frozen at -22°C.

From the observations just recorded it is plain that the constituent of normal tissues with which the natural antibody reacts is either inactivated by alcohol or does not come away into it. It is destroyed by mild heating (56-70°C. for 30 minutes), its effectiveness diminishes upon standing a few days in the refrigerator, and it is adversely affected by glycerol and by certain salts. Further consideration will be given to its properties in an associated paper.

SUMMARY

The foregoing experiments have shown that complement fixation takes place when the blood serum of normal adult rabbits is mixed with fresh saline ex-

tracts of normal rabbit tissues under controlled conditions. A natural antibody, which reacts *in vitro* with a sedimentable constituent of normal tissue cells, is responsible for the phenomenon.

Further observations on the theme are reported in an associated paper, and the findings as a whole are discussed.

References to both papers are given at the end of the second paper (page 576).

A NATURAL ANTIBODY THAT REACTS IN VITRO WITH A SEDIMENTABLE CONSTITUENT OF NORMAL TISSUE CELLS

II SPECIFICITY OF THE PHENOMENON GENERAL DISCUSSION

By JOHN G KIDD, M.D., AND WILLIAM F FRIEDEWALD M.D

(From the Laboratories of The Rockefeller Institute for Medical Research)

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In the preceding paper experiments are described which demonstrate the presence of a natural antibody in the blood of normal adult rabbits which reacts with a sedimentable constituent of normal rabbit tissue cells. Observations will now be reported which deal further with the distribution and affinities of antigen and antibody and with their characters.¹

Reaction of the Natural Antibody and Various Rabbit Tissue Extracts

In the foregoing experiments, saline extracts of various rabbit tissues nearly all reacted with sera containing the natural antibody, though there were noteworthy and fairly regular differences in their capacity to do so (Table VI). It seemed possible that the quantitative differences might be due either to a single substance present in various amounts in the different tissue cells which reacts with a single natural antibody, or to a variety of organ- or cell-specific substances each reacting with its own natural antibody. In an experiment to learn more about this, the sera of 8 normal rabbits of various breeds were tested in mixture with antigens made from the freshly procured kidney, liver, spleen, brain, and heart muscle tissues of a single rabbit. Table X shows the results of the tests, which confirm and extend those of the preceding experiments. As in the tests of Table VI, the various sera manifested regular differences in their abilities to react with the several antigens, in general fixing complement best in mixture with the kidney antigen, next best with the one made from liver, and then with those derived from spleen, brain, and heart muscle, in the order named. The serum with the highest titer of the natural tissue antibody, as shown by its ability to react with the kidney and liver antigens—that of rabbit 69—also reacted best with the spleen, brain, and heart muscle antigens. And so too, in diminishing titers, did the sera of rabbits 63, 65, and 66. The rest of the sera (70, 74, 77, and 71) exhibited progressively weaker reactions in mixture with the kidney antigen. They failed to react perceptibly with the heart antigen and gave weak fixation or none at all in mixture with the brain, spleen, and liver antigens.

¹ To facilitate discussion the tables and references in the two papers are numbered consecutively.

The regularity of the findings just described would seem to suggest that the natural tissue antibody has a specific affinity for a single substance, which is present in various amounts in different tissues. Further evidence that this is the case is provided by the findings of the next experiment, in which sedimented substances derived from normal rabbit kidney, brain, and liver, respectively, were used in absorption tests with sera known to contain the natural antibody.

The sera were first heated at 56°C for 15 minutes to inactivate complement, and then were absorbed with washed sheep erythrocytes (2.0 cc of packed cells plus 6.0 cc of each serum, incubated 15 minutes at 37°C) to remove any natural sheep hemolysin.

Complement Fixation

Source of serum											
Rabbit No	Breed of rabbit	Kidney					Liver				
		Serum dilution					Serum dilution				
		1 2	1 4	1 8	1 16	1 32	1 2	1 4	1 8	1 16	
69	New Zealand	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
63	Agouti hybrid	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
65	" "	++++	++++	++++	++++	+++±	++++	++++	++++	++++	++++
66	New Zealand	++++	++++	++++	++++±	±±	++++	++++	++++	++++	+
70	Chinchilla	++++	++++	++++	++	0	++++	+	0	0	0
74	Blue Cross hybrid	++++	+++±	±±	0	0	++	0	0	0	0
77	" " "	++++	+++±	0	0	0	0	0	0	0	0
71	Chinchilla	+++	±±	0	0	0	0	0	0	0	0

The antigens were 1:30 saline extracts made as usual from the fresh normal tissues of a single animal.

The kidney, brain, and liver particles to be used for absorption were prepared by grinding the fresh tissues of a single rabbit, suspending these 1:10 in saline and centrifuging at 4400 R.P.M. for 10 minutes. The supernatant fluids were then spun at 25,000 R.P.M. for 1 hour in the air-driven machine, and the sedimented materials resuspended in one-third the original volume of dilute phosphate buffer, pH 7.3. The suspensions of kidney and liver particles were densely opalescent, that of the brain particles much less so. Just before use all of the suspensions were rendered isotonic by the addition of suitable amounts of 18 per cent NaCl solution. Mixtures of the four sera were made with 3 volumes of saline, and with 3 volumes of the 1:10 suspensions of kidney, brain, and liver particles, respectively. The absorption mixtures were incubated 2 hours at 37°C, then kept overnight in the refrigerator. None developed visible floccules. They were all spun at 25,000 R.P.M. for 1 hour and the supernatant liquids removed for test with antigens consisting of 1:30 saline extracts made as usual from the frozen kidney, brain, and liver tissues of another rabbit.

Table XI shows the results of the tests. All of the control mixtures of sera and saline reacted with the three antigens in varying degrees, whereas the specimens that

had been absorbed with the kidney substance failed to do so. The sera that had been absorbed with the brain material had no capacity to react with the brain and liver antigens and their ability to react with the kidney antigen was markedly reduced. The liver substance had absorbed completely the capacity to react with liver antigen and largely that to react with brain antigen, and had brought about a marked reduction in the reactions with the kidney antigen.

Manifestly some antibody remained in the mixtures absorbed once with the brain and liver materials for these retained in some part their capacity to react with the kidney antigen. To learn whether this residual antibody could be removed, the specimens of rabbits 15-88 and 15-90 that had been absorbed once with brain substance were again absorbed as before this time with an equal volume of a 1:10 suspension of

Rabbit Sera and Antigens

ANTIGENS

Spleen				Brain					Heart muscle				
Serum dilution				Serum dilution					Serum dilution				
4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32
+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	±
+++	+++	+++	0	+++	+++	+++	++	0	+++	+++	++	±	0
+++	+++	±	0	+++	+++	++	0	0	+++	+	0	0	0
0	0	0	0	±	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0

5)

rabbit liver substance prepared as previously from the frozen tissues of the same animal, and likewise the specimens of rabbits 5-74 and 15-84 that had been absorbed first with liver were reabsorbed with a fresh preparation of brain. Subsidiary tests were then made to see whether the twice absorbed sera, along with appropriate saline controls, would still react with a normal rabbit kidney antigen. The controls did so precisely as in the table but the reabsorbed sera gave no reactions whatever.

In sum, the various tissue substances differed notably in their capacity to absorb the natural antibody and to fix complement in mixture with it, but there is no suggestion that these differences were more than quantitative (Table XI). On the contrary, the kidney substance (which was the most potent) absorbed completely the capacity of the sera to react with all of the antigens, and repeated absorption with either brain or liver substance (which were less potent) had the same effect. The findings have been repeatedly confirmed in other similar experiments.

TABLE XI
Absorption of the Natural Antibody with Suspensions of Sedimented Substances from Rabbit Kidney, Brain, and Liver

Normal rabbit (sera)	Absorbed with*	Complement fixation tests															
		Kidney antigen				Brain antigen				Liver antigen							
		Serum dilution				Serum dilution				Serum dilution							
		1 4	1 8	1 16	1 32	1 64	1 4	1 8	1 16	1 32	1 64	1 4	1 8	1 16	1 32	1 64	
5 74	Nil, saline control	++	++	++	++	0	++	++	0	0	0	++	++	+	0	0	
15-84	"	++	++	++	++	0	++	++	++	0	0	++	++	++	0	0	
15 88	"	++	++	++	++	0	++	++	++	0	0	++	++	0	0	0	
15 90	"	++	++	++	++	0	++	++	++	0	0	++	++	0	0	0	
5 74	Kidney substance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
15 84	"	0	0	0	0	0	±	0	0	0	0	0	0	0	0	0	
15 88	"	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
15 90	"	0	0	0	0	0	±	0	0	0	0	0	0	0	0	0	
5-71	Brain substance	++	++	0	0	0	0	0	0	0	0	0	0	0	0	0	
15 84	"	++	++	++	++	0	0	0	0	0	0	0	0	0	0	0	
15-88	"	++	++	++	++	0	0	0	0	0	0	0	0	0	0	0	
15 90	"	++	++	++	++	0	0	0	0	0	0	0	0	0	0	0	
5-74	Liver substance §	++	++	±	0	0	±	0	0	0	0	0	0	0	0	0	
15 84	"	++	++	++	++	0	++	++	0	0	0	0	0	0	0	0	
15-88	"	++	++	++	++	0	++	0	0	0	0	0	0	0	0	0	
15 90	"	++	++	++	++	0	++	±	±	0	0	0	0	0	0	0	

Antigens, 1-30 saline extracts of frozen normal rabbit tissues from a single normal rabbit.

* See text for description of method and materials

† Negative after reabsorption with brain substance, —see text.

§ Negative after reabsorption with liver substance, —see text

Reaction of the Natural Rabbit Antibody with Extracts of Tissues of Alien Species

The fact that the substance with which the natural antibody reacts can be extracted from many rabbit tissues (Tables VI and X) brings up the possibility that it may be present also in the tissues of other species. This proved to be the case. Tests were made using rabbit sera known to contain the natural antibody in mixture with saline extracts of the liver, kidney and brain of the mouse, guinea pig, chicken, and rat. Fixation was invariably got, and the reactions were about as potent as those with extracts of corresponding rabbit organs. As a further step towards identifying the affinities of the natural antibody, it seemed important to learn whether absorption of rabbit sera with sedimented substances from rabbit tissues would remove their capacity to react with extracts of the tissues of alien species.

The results of an experiment to test the question are set down in Table XII, in which the sera of two domestic rabbits were absorbed precisely as in the experiment of Table VI (q v) with sedimented substances derived from rabbit kidney and liver. The absorbed sera, along with control unabsorbed specimens, were tested with antigens made from the frozen kidney, liver and brain tissues of various normal animals (rabbit, mouse, guinea pig, chicken and rat). Both of the unabsorbed specimens gave fixation in mixture with all of the tested antigens (the chicken brain antigen was omitted through an oversight). As was expected from the findings already mentioned, fixation was about as potent with antigens procured from the alien species as with those from the rabbit, though the rat kidney antigen was notably more potent in this experiment for some reason not understood. Absorption with the sedimented substance from normal rabbit kidney rendered both sera devoid of capacity to react with any of the antigens, except for slight reactions in the mixtures containing rat and mouse kidney antigens. Absorption with the liver substance which as in the preceding experiment, was less potent as an absorbing material than the kidney substance, also removed largely or completely the capacity of the sera to react with all of the antigens, whether derived from rabbit tissues or from those of the other species.

Absorption with sedimented substances from normal rabbit tissues removed not only the capacity of rabbit sera to react with extracts of homologous tissues, but also their ability to react with extracts of the tissues of alien species (Table XII). It follows that the tissue substance with which the natural antibody reacts is probably the same, or much the same, whether derived from rabbit tissues or from those of other species.

The Natural Tissue Antibody As Distinct from Those Directed Specifically against the Rabbit Papilloma Virus and the Distinctive Substance of the Brown Pearce Tumor, Respectively

In previous serological studies with the rabbit papilloma virus (6) and the distinctive substance of the Brown-Pearce tumor (1), the test antigens were prepared as routine from glycerolated (or occasionally frozen) tissues. These

TABLE XII
Complement Fixation Reactions with Normal Rabbit Sera and Tissue Antigens from Other Species

Normal rabbit sera	Absorbed with	Source of antigen	Kidney antigens					Liver antigens					Brain antigens				
			Serum dilution					Serum dilution					Serum dilution				
			1 8	1 16	1 32	1 64	1 128	1 8	1 16	1 32	1 64	1 128	1 8	1 16	1 32	1 64	1 128
15-88	Nil, saline control	Rabbit	++++	++++	+++	0	0	+++	+	0	0	0	+++	0	0	0	0
		Mouse	++++	++++	+	0	0	++	+	0	0	0	++++	+	0	0	0
		Guinea pig	++++	++++	+	0	0	++	0	0	0	0	++++	+	0	0	0
		Chicken	++++	++++	0	0	0	+++	0	0	0	0	++++	+	0	0	0
	Sedimented substance from rabbit kidney	Rat	++++	++++	++++	++++	0	++++	+	0	0	0	++++	+	0	0	0
		Rabbit	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Mouse	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Guinea pig	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sedimented substance from rabbit liver	Chicken	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Rat	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Rabbit	+++	+	0	0	0	0	0	0	0	0	0	0	0	0	0
		Mouse	+++	+	0	0	0	0	0	0	0	0	0	0	0	0	0
15 90	Nil, saline control	Guinea pig	+++	0	0	0	0	0	0	0	0	0	+	0	0	0	0
		Chicken	+++	0	0	0	0	0	0	0	0	0	+	0	0	0	0
		Rat	+++	+++	+++	0	0	+	0	0	0	0	+	+	0	0	0
		Rabbit	+++	+++	+++	0	0	+	0	0	0	0	+	+	0	0	0
	Sedimented substance from rabbit kidney	Mouse	+++	+++	+++	0	0	+	0	0	0	0	+	+	0	0	0
		Guinea pig	+++	+++	+++	0	0	+	0	0	0	0	+	+	0	0	0
		Chicken	+++	+++	+++	0	0	+	0	0	0	0	+	+	0	0	0
		Rat	+++	+++	+++	0	0	+	0	0	0	0	+	+	0	0	0
	Sedimented substance from rabbit liver	Rabbit	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Mouse	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Guinea pig	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Chicken	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Both sera had first of all been heated at 56°C for 30 minutes and absorbed with clean autogenous (a) serum.

were extracted overnight in the refrigerator, then spun clear, and heated at 56°C for 30 minutes immediately prior to use—thus following the time-honored, empirical procedures to avoid or diminish “non specific” complement fixation. The findings of the present work have shown that the substance with which the natural tissue antibody reacts is adversely affected by glycerol, that it is largely inactivated upon heating at 56°C for 30 minutes, and that it deteriorates rapidly when left in the refrigerator in suspension in isotonic saline. The procedures employed empirically in the preceding work were as though designed to destroy the tissue constituent with which the natural antibody reacts. Hence it is not surprising that the natural antibody remained undisclosed in the control tests of those studies.

It is interesting to note in this connection that the reactions of the natural tissue antibody and the sedimentable constituent of normal tissues were not observed by Hoyle (7) or by Cheever (8), who partially repeated the serological studies with the papilloma virus and the Brown Pearce tumor antigen respectively. Jacobs and Houghton (9) on the other hand while attempting to repeat the serological experiments with the Brown Pearce tumor, observed that some normal sera had the capacity to fix complement under the conditions of their tests, and that prolonged centrifugation or filtration through a Mandler filter or simply passage of time would reduce the complement fixing capacity of their antigens. It seems unlikely that they encountered any sera containing the antibody that reacts specifically with the distinctive Brown Pearce tumor antigen for the reactions they observed were weak at best. The interpretation of their findings, which are not given in detail, must remain in doubt but it is possible that reactions of the natural antibody and the normal tissue substance may have been responsible for the results.

With a view to learning whether the natural tissue antibody may have entered into the reactions already reported as involving the papilloma virus (6) and the Brown Pearce tumor antigen (1), an experiment was set up in which specimens of the sera of normal rabbits, of rabbits carrying virus papillomas, and of rabbits implanted with the Brown-Pearce tumor were heated at 56°C and at 65°C and tested for capacity to fix complement in mixture with various antigens.

The extracts containing the antigens were made fresh from frozen tissues, as in the experiments of the present paper. Table XIII shows the results of the experiment the details follow—

The normal rabbit sera (5-65 and 5-69) were known from previous tests to contain the natural tissue antibody in high titer. Rabbits 14-71 and 14-72 carried four large pancake papillomas as result of the inoculation 42 days before of highly pathogenic W.R. (wild cottontail rabbit) 128 virus. The sera of rabbits 5-01 and 5-03 had been procured 31 days following multiple implantations with the Brown Pearce tumor they had been much used in the Brown Pearce work already reported and were known to contain the specific antibody in high titer (1). Specimens of the sera were heated

TABLE XIII
Tests for Natural Tissue Antibody and for the Specific Antibodies Elicited by Virus Papillomas and the Brown-Pearce Carcinoma

SERA			ANTIGENS															
Heated (30 min.)	Procured from	Rabbit No	Normal rabbit kidney					Virus papilloma—W R					Brown Pearce carcinoma					
			Serum dilution					Serum dilution					Serum dilution					
	(a) Normal rabbits	5-65 5-69	1 4	1 8	1 16	1 32	1 64	1 4	1 8	1 16	1 32	1 64	1 4	1 8	1 16	1 32	1 64	
56°C	(a) Normal rabbits	5-65 5-69	+++++	+++++	+++++	+++++	+++	0	0	0	0	0	+++++	+++++	+++++	+	0	
	(b) Rabbits carry- ing virus pap- illomas	14 71 14 72	+++++	+++++	+++++	+++++	+++	+++++	+++++	+++++	+++++	+++	+++++	+++	0	0	0	
	(c) Rabbits im- planted with the Brown Pearce car- cinoma	5-01 5-08	+++++	+++++	+++	0	0	0	0	0	0	0	+++++	+++++	+++++	+++++	+++++	
65°C	(a) Normal rabbits (as above)	5-65 5-69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	(b) Rabbits carry- ing virus pap- illomas	14 71 14 72	0	0	0	0	0	+++++	+++++	+++++	+++++	++	0	0	0	0	0	
	(c) Rabbits im- planted with the Brown Pearce car- cinoma	5-01 5-08	0	0	0	0	0	0	0	0	0	0	+++++	+++++	+++++	+++++	+++++	

Antigens, 1:40 saline extracts of frozen tissues

at 56°C. and at 65°C. and tested in mixture with antigens made precisely as in the other experiments of this paper: fresh 1:40 saline extracts of frozen tissues from (a) normal rabbit kidney (D.R. 4-64), (b) a naturally occurring virus papilloma (W.R. K. 1), and (c) a vigorously growing Brown Pearce tumor from the leg muscles of D.R. 14-04.

From Table VIII it will be seen that all of the six sera contained the natural tissue antibody, for, when heated at 56°C., all fixed complement in mixture with the normal rabbit kidney antigen, but when heated at 65°C., all failed to do so. As already stated the two normal rabbit sera had been chosen because they were known from previous tests to contain the natural tissue antibody in high titer. The titer of this was lower in the sera of the rabbits carrying virus papillomas and still lower in the sera of the rabbits carrying Brown Pearce carcinomas, as the table shows, but this would seem to be merely fortuitous. It is interesting that the sera of the normal rabbits and those of the Brown-Pearce rabbits gave no fixation or practically none in mixture with the virus papilloma antigen. Manifestly the tissue substance with which the natural antibody reacts was not present in effective amount in the 1:40 saline extract of the virus papilloma. The normal rabbit sera heated at 56°C. gave positive reactions in dilutions as high as 1:16 in mixture with the frozen Brown Pearce tumor antigen and so too in lesser dilutions did the sera of the rabbits carrying virus papillomas. But the reactions were of an entirely different order in the mixtures containing the sera of rabbits implanted with the Brown Pearce carcinoma (5-01 and 5-08), in these fixation was complete in all dilutions tested.

The real and striking specificity of the serological reactions is brought out in the results with the mixtures containing the six serum specimens heated at 65°C. for the natural tissue antibody was thus destroyed. The sera of the normal rabbits gave no reaction with any of the three antigens: those of the rabbits carrying virus papillomas (14-71 and 14-72) reacted to considerable dilutions with the virus papilloma antigen but failed to fix complement in mixture with the normal kidney or Brown-Pearce tumor antigens and the sera of the rabbits implanted with the Brown-Pearce tumor (5-01 and 5-08) reacted with the Brown Pearce tumor antigen in all of the dilutions tested but gave no fixation in mixture with either of the other two antigens. There were no cross-reactions whatever.

From the findings just given (Table XIII) and those already recorded it becomes plain that the natural antibody and the normal tissue constituent with which this reacts can be largely disregarded in complement fixation tests with the papilloma virus and its antibody. For the normal tissue substance cannot be detected in the dilutions of papilloma extracts employed as routine, while furthermore, as already stated, glycerol inactivates the normal tissue substance, and glycerolated papillomas have been regularly employed as a source of antigen in complement fixation tests with the papilloma virus and its antibody. In addition, an extensive experience has now shown that the complement fixing capacity of any given papilloma virus-immune serum invariably parallels its virus-neutralizing capacity, and many findings indicate that the antiviral antibody reacts with the virus itself or with an integral part thereof, and that it

has no affinity for other constituents of the papilloma cells nor any for normal tissue components (6)

The circumstances differ, however, with respect to serological experiments with extracts of the Brown-Pearce tumor. For, as Table XIII shows, normal rabbit sera that contain the natural antibody will react with freshly made extracts of frozen Brown-Pearce tumors, which would seem to contain a small quantity of the "normal" tissue constituent in addition to the tumor-specific one². True, this reaction is not notably strong, even when sera containing high titers of the natural antibody are employed, and it can be avoided if the sera are heated at 65°C for 30 minutes,—an amount of heating that has no significant effect upon the antibody that reacts specifically with the Brown-Pearce tumor antigen (Table XIII). Repeated experiments have shown, furthermore, that the reactions with normal sera can also be avoided if the Brown-Pearce tumor antigens are prepared as in the original work (1),—i.e., by allowing the saline extracts to stand overnight in the refrigerator, then centrifuging and heating at 56°C for 30 minutes prior to use. For in this way, as already mentioned, the tissue constituent with which the natural antibody reacts is inactivated. Even so, it has seemed essential to resurvey the question of the specificity of the serological reactions with extracts of the Brown-Pearce tumor. This has recently been done and the results will soon be published in detail³.

Strict Affinity of the Natural Antibody for the Sedimentable Tissue Constituent

In order to learn more about the specificity of the reaction between the natural antibody and the sedimentable constituent of normal tissues, an experiment was next set up in which rabbit sera known to contain various specific antibodies and presumably the natural antibody as well were tested for capacity to fix complement in mixture with antigens made from normal rabbit liver,

² More will be said about the presence of the "normal" tissue constituent in extracts of various diseased tissues in connection with the next experiment and in later communications.

³ Briefly, the findings support the conclusions reached heretofore (1). The antibody that reacts specifically with the distinctive constituent of the Brown-Pearce tumor develops infrequently in ordinary hybrid rabbits implanted with the growth, as Cheever noted (8), yet it often reaches high titer in specially favorable hosts, as in the sera of rabbits 5-01 and 5-08 of Table XIII. It has never been found in the serum of normal rabbits, in that of rabbits carrying transplanted cancers of other sorts, or in the blood of control animals with syphilis or other laboratory infections. The distinctive substance with which it reacts is regularly present in large amounts in the Brown-Pearce tumor, but cannot be detected in extracts of the normal tissues of rabbits or in other rabbit neoplasms.

from the Brown Pearce tumor, and from virus-induced fibromas, vaccinia lesions, and virus papillomas respectively

Five sera were used. That of normal rabbit 6-72 was known from previous tests to contain a high titer of the natural antibody. Rabbit 5-52 had been implanted in six muscle situations with the Brown Pearce tumor 46 days before bleeding; tumors appeared at all of the implanted situations and reached a size of 3.0 to 5.0 cm. across before the 18th day but regressed abruptly between the 18th and the 28th days. The serum of this rabbit had been found on previous tests to contain a high titer of the specific antibody for the distinctive substance of the Brown Pearce tumor; it had not been tested for the natural antibody. Rabbit 1-74 had been used to titrate the fibroma virus; this had been inoculated into the skin of the rabbit at 24 situations, with result in as many large fibroma lesions which had healed when the animal was bled 38 days after the inoculations. The serum of rabbit 16-01 had come from an animal originally infected with vaccine virus, which had later received repeated injections of suspensions of the elementary bodies of vaccinia. It was generously made available by Dr. Joseph E. Smadel. Rabbit 16-41, which provided the final serum, had received three intraperitoneal injections of a filtrate containing large amounts of the papilloma virus. Its serum was known to contain the specific papilloma virus antibody in high titer.

The five antigens came from normal rabbit liver, the Brown Pearce tumor, and from rabbit tissues infected respectively with the fibroma virus, vaccine virus, and the papilloma virus. The swollen testicles of a rabbit infected 3 days previously with fibroma virus had been saved frozen, as had also the normal liver and Brown Pearce tumors of other animals. The glycerolated natural papillomas of W.R. 2-95 provided an antigen known to be rich in papilloma virus. The frozen liver, Brown Pearce tumor and fibroma tissues, and the glycerolated W.R. papilloma tissue were ground as usual and suspended 1:10 in dilute phosphate buffer. The suspensions were spun at 4400 R.P.M. for 10 minutes to remove coarse tissue fragments and the supernatant fluids then spun at 25,000 R.P.M. for 1 hour in the high-speed centrifuge. The clear supernatant liquids were poured off and the sedimented pellets resuspended in the original volume of dilute buffer for use in the tests. Dr. Smadel generously furnished the fifth antigen, a potent suspension of the elementary bodies of vaccinia freshly prepared by a standard method (10).

Table XIV shows the results of the tests. All of the sera except one (1-74) contained the natural antibody in considerable titer, as shown by the reactions with normal liver antigen of the specimens heated at 56°C. which were abolished when the sera were heated at 65°C. Normal serum 6-72 also reacted with the Brown Pearce tumor antigen and with the fibroma antigen, but much less well with these than with the normal liver antigen, and so too with the three other sera that contained the natural antibody (5-52, 16-01, 16-41). It is especially noteworthy that none of the sera reacted with the purified suspension of vaccinia elementary bodies except that of D.R. 16-01, which contained specific, heat-stable antivaccinia antibodies, and likewise that none of the sera reacted with the antigen containing the papilloma virus except that of D.R. 16-41, which contained the specific papilloma virus antibody. As already mentioned, the reactions of the natural antibody were abolished when the sera were heated at

65°C for 30 minutes, whereas the specific antibodies,—those directed respectively against the distinctive constituent of the Brown-Pearce carcinoma (serum 5-52), the fibroma virus or an associated soluble antigen (serum 1-74), the L-S complex of vaccinia (serum 16-01), and the papilloma virus (serum 16-41),—were but little affected

Tests for Natural and

ANTIG

SERA			Normal rabbit liver						Brown Pearce tumor					
Heated (30 min)	Rabbits	Rab bit No	Antigen dilution						Antigen dilution					
			1 10	1 20	1 40	1 80	1 160	1 320	1 10	1 20	1 40	1 80	1 160	
56°C	Normal	6-72	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	
	With Brown Pearce tumor	5-52	++++	++++	++++	+++	±	0	++++	++++	++++	++++	++++	
	Immune to fi broma virus	1 74	0	0	0	0	0	0	0	0	0	0	0	
	Hyperimmune to vaccinia	16-01	++++	++++	++++	+++	+	0	++++	+++	++	0	0	
	Hyperimmune to pap virus	16-41	++++	++++	++++	++++	++++	+++	++++	++++	++++	+++	++	
65°C	Normal	6-72	0	0	0	0	0	0	0	0	0	0	0	
	With Brown Pearce tumor	5 52	0	0	0	0	0	0	++++	++++	++++	++++	++++	
	Immune to fi broma virus	1 74	0	0	0	0	0	0	0	0	0	0	0	
	Hyperimmune to vaccinia	16-01	0	0	0	0	0	0	0	0	0	0	0	
	Hyperimmune to pap virus	16-41	0	0	0	0	0	0	0	0	0	0	0	

Antigens All spun at 4400 R.P.M for 10 minutes to remove coarse material, then at 25,000 R.P.M for 10 minutes to remove fine material. The antigen was a suspension of elementary bodies prepared according to a standardized method.

Sera All 1 8

The results of the experiment (Table XIV) show clearly that the heat-labile natural antibody, which was present in substantial amount in four of the five sera (all except 1-74), failed to react with antigens containing large amounts of vaccine virus and papilloma virus respectively, though it reacted notably well with the antigen made from normal rabbit liver and to a lesser extent with those derived from the Brown-Pearce tumor and the fibroma lesions. Manifestly, the natural antibody has a strict affinity for the sedimentable tissue constituent, which is present in extracts of certain diseased as well as normal tissues, and none for the two viruses mentioned.

GENERAL DISCUSSION

The experiments here described took origin from the observation that the blood serum of normal adult rabbits will fix complement when mixed with

ious Rabbit Sera

roma

roma			Vaccinia lesion						Virus papilloma—W.R.					
dilution			Antigen dilution						Antigen dilution					
1:40	1:80	1:160	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0
+++±	±±	0	0	0	0	0	0	0	0	0	0	0	0	0
+++±	0	0	0	0	0	0	0	0	0	0	0	0	0	0
+++	±	0	++++	++++	++++	++++	±	0	0	0	0	0	0	0
++++	+++±	0	0	0	0	0	0	0	++++	++++	++++	++++	++++	++++
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
±±	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	++++	++++	++++	+++	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	++++	++++	++++	++++	++++	++++

nalson of the sedimented pellets in the original volume of dilute phosphate buffer,—except the made available by Dr Smadel.

saline extracts of normal tissues. Facts were procured which show that the reaction is due to a naturally occurring serum principle which combines specifically *in vitro* with a sedimentable constituent of many normal tissue cells. Both the serum principle and the substance with which it reacts have implications for discussion.

The serum principle would seem to be a natural antibody. For, as the experiments have shown, it appears naturally in the blood of almost all normal adult rabbits, and, like the generality of antibodies, can be precipitated from serum with ammonium sulfate, fixes complement in mixture with a single sub-

stance (or class of similar substances), and can be specifically absorbed thereby in the absence of complement. Furthermore it resembles other natural antibodies of normal rabbit's blood in being more labile to heat than are induced antibodies. Yet to state that the serum principle would seem to be a natural antibody is not to define it precisely, for the character of these is but poorly understood.

Origin and Character of the Natural Tissue Antibody

The natural tissue antibody manifestly originates under physiological conditions. It could be an autoantibody, meaning thereby that it might appear in response to constituents of normal tissue cells that act as antigens following liberation from injured or dying cells. Or it might be formed in a simpler way—the synthesis of globulin might normally take place—as implied in the theories of Haurowitz, Mudd, and others (12)—in such a way that some of the molecules would become specifically oriented to conform with a certain part of the sedimentable cell constituents, with result in a fraction of “normal” globulin having the special affinity that distinguishes what has here been termed the natural tissue antibody. Rabbit globulin as such does not manifest its properties, however, for the quantity of normal globulin differs but little from one adult rabbit serum to the next, as is well known, whereas the titer of the natural antibody varies quite widely. In this connection, the fact may be mentioned that a number of rabbit sera were recently tested for capacity to flocculate mastic—a reaction presumably brought about by normal globulin (11)—and, at the same time, for capacity to fix complement in mixture with normal tissue extracts. The various sera proved almost precisely alike in their ability to flocculate the gum but differed markedly in their reactions with extracts of normal tissue.

The natural tissue antibody would seem to be in a different category from certain other so called natural antibodies,—the ones effective respectively against herpes, yellow fever, and influenza viruses, *E. coli*, the toxins of scarlet fever and diphtheria, for example. For these latter are hardly “natural” in a strict sense but appear to represent responses to chance contacts with the viruses or with the specific microorganisms or their products (13). Furthermore, they have to do in part at least with the development of immunity to the agents mentioned (13), whereas the natural tissue antibody has no discernible relation to the phenomena of immunity to disease.

The natural tissue antibody is distinguishable by its strict affinity for a sedimentable substance extractable from many normal tissue cells. It is not absorbed by sheep erythrocytes, as are the natural Forssman antibodies, and it does not react with alcoholic tissue extracts, as do the natural Wassermann antibodies of rabbit's blood. Furthermore it differs from other so called physiological antibodies in that both it and the substance with which it

reacts are usually present together in the same individual, while the antibodies that react with the blood group substances, for example, and those that react with the Forssman substances are present only in individuals with tissues presumably devoid of those substances (14)

At first thought it seems strange that an individual can carry in its blood an antibody capable of reacting with a constituent of its own tissues. Indeed one might suppose that under such circumstances the antibody would necessarily be absorbed and become fixed upon the tissue components for which its affinities fitted it, as actually happens in the paroxysmal hemoglobinuria of syphilitic human beings, in which induced autoantibodies cause lysis of erythrocytes. But this reaction takes place only at temperatures somewhat lower than those normal for the human body (15), and so too when the natural and induced autohemagglutinins of rabbit's blood cause clumping of red cells (16). By contrast the antibody now under consideration reacts at 40°C. and quite as well as at lower temperatures, as recent experiments have shown. Hence other reasons must be sought to explain why it fails to react *in vivo* with the normal tissue constituent.

The natural antibody, once it gets into the blood, may never again come into effective contact with the sedimentable constituents of living cells. This would be true for example, if the latter were situated within the cell protoplasm or were associated with other cell constituents in such wise as to be shielded. In this relation the fact may be recalled that viruses are protected from the action of specific antiviral antibodies so long as they remain associated with living cells,—a phenomenon that is especially striking in the case of the rabbit papilloma virus, which continues to increase in amount in association with proliferating cells even when these are constantly nourished by blood having great antiviral power (17). The circumstances may be similar also with respect to the substance (or substances) that reacts with Wassermann reagin. The tissues of syphilitic individuals will yield the Wassermann substance as readily as those of normal ones, yet the reagin that circulates in their blood is not demonstrably absorbed *in vivo*. In the light of these findings it scarcely seems surprising that the natural tissue antibody apparently fails to react *in vivo* with the normal tissue substance, and it need not seem strange that the antibodies induced in rabbits against sedimentable kidney substances from alien species fail to exhibit nephrotoxic properties though capable of reacting *in vitro* with sedimentable constituents of rabbit kidney cells (18).

Natural Antibodies and Antigens in Relation to "Non-Specific" Complement Fixation and to Other Serological Complexities

It is obvious that the natural tissue antibody may complicate the results of serological experiments in which rabbit sera are used in mixture with tissue extracts. Since natural antibodies of the same or similar sort may be present in other species (as the sedimentable tissue substances unquestionably are (5)) it seems possible that reactions between them may be responsible for some at least of the so called "non-specific" complement fixation reactions encountered in the past.

The literature on "non-specific" complement fixation reactions has been dealt with by Noguchi (19) and Gussenbauer (20), in various text books (21), and recently by Takenomata (22), Mackie and Finkelstein (23), and Gibson (24). In addition to anticomplementary effects due to chemical inactivation or destruction or adsorption of complement, there are other, so called non specific, complement fixation reactions in which substances present in the serum of normal animals and man will fix complement in mixture with a variety of "non-antigenic" substances, amongst them peptone, glycogen, various extracts of tissues and bacteria, amino acids, cholesterol, and alcohol.

Many workers have observed that certain "non-specific" complement fixation effects can be more or less readily avoided by empirical means. This has proved true also in the case of the natural tissue antibody. As already mentioned (experiment of Table XIII), certain tissue antigens can be prepared, or diluted, in such a way that reactions with the natural antibody are avoided. The same purpose can often be achieved by diluting the serum. Only occasionally have sera containing the natural tissue antibody been effective in dilutions beyond 1/16 or 1/32, in our experience, whereas the antibodies appearing in rabbit serum in response to foreign antigens often titer much higher, as is generally known.

Still another method long used empirically to avoid "non-specific" complement fixation is to heat the sera to such an extent that the non-specific effects are abolished while the specific antibodies are left unaffected. Noguchi (19), Kolmer (25), and Gibson (24) made much use of this procedure, and Casals and Palacios have recently applied it with noteworthy success in complement fixation tests with various neurotropic viruses and their antibodies (26). The findings of the present work provide a rational basis for the use of heat for this purpose. For they show that heating at 65°C for 30 minutes destroys the natural antibodies in normal rabbit serum without destroying specific, induced ones. In the experiments of Tables XIII and XIV recourse was had to this procedure to bring into sharp relief the specific reaction between the distinctive substance of the Brown-Pearce tumor and its antibody and other specific antigen-antibody reactions as well.

Duran-Reynals has reported upon a flocculation of tissue extracts by normal and immune sera of fowls and other animals (27). The phenomenon differs notably, however, from that here dealt with. For the flocculations took place only at low temperatures, they occurred when alcoholic as well as saline tissue extracts were employed, the titer of the serum factor responsible for them could be markedly raised by immunization with specific antigens, and the latter proved capable of absorbing concurrently both the non-specific flocculating factor and the specific antibodies.

Many studies attest to the complexity of serological reactions with the fowl tumor agents, and to the close association of these agents with normal tissue constituents (28). In particular it has been shown by numerous workers (28) that the sera of normal adult fowls will not infrequently neutralize the causative agent of Chicken Tumor I, and Andrewes and Amies have observed cross-reactions when the sera of fowls carrying various tumors were mixed with tissue suspensions containing the filtrable agents responsible for them. It is conceivable that natural antibodies of the sort disclosed in the present study or induced ones with similar affinities might be responsible for some of the puzzling reactions, by acting upon normal tissue constituents

with which the disease producing agents may be associated. The possibility would seem to be brought nearer by the findings of various workers (28) who have noted that the sera of rabbits and goats injected repeatedly with normal fowl tissue will neutralize the filtrable agent responsible for Chicken Tumor I, and also by observations of Chambers and Henle, who have recently shown that antisera prepared by injecting mouse lung particles into rabbits possess the capacity to agglutinate particles of the sort injected as also to carry down influenza virus Type A when this is present, presumably in association with the normal tissue constituents (29).

Reference has already been made to the fact that the natural antibody can be disregarded in complement fixation tests in which the rabbit papilloma virus and its antibody are concerned (see the experiments of Tables XIII and XIV), for the reason that the "normal" tissue constituent is not present in detectable amounts in papilloma extracts employed as antigen in such tests. It seems likely, however, especially in view of the work of Casals and Palacios already cited (26), that *in vitro* reactions of the natural antibody and the normal tissue constituent may complicate serological tests with other viruses. This actually happened in the experiment of Table XIV in which normal rabbit sera, containing the natural tissue antibody, reacted with an antigen made from tissues infected with fibroma virus, even though the antigen had been purified by differential ultracentrifugation. It follows that the natural antibody may prove useful as an indicator of the presence of "normal" tissue constituents in virus preparations purified in this way, for, as will be discussed further on, the substance with which it reacts is thrown down in the high-speed centrifuge just as viruses are. In the experiment of Table XIV, the suspension of vacuinal elementary bodies purified by Dr. Smadel was apparently devoid of the normal tissue constituent, and so too was the preparation containing purified papilloma virus, for these did not react with the sera known to contain the natural antibody. It is noteworthy that both viruses were procured from epidermal cells, which have been found to contain comparatively little of the normal tissue constituent with which the natural antibody reacts.

The Constituent of Normal Tissue Cells with Which the Natural Antibody Reacts

An outstanding property of the tissue constituent is that it can be thrown down readily in the high speed centrifuge, little or none remaining in the supernatant liquid of potent suspensions spun for an hour at speeds of 20,000 to 30,000 R.P.M. (about 29,000 to 65,000 times gravity), as Table VII shows.

Substances readily sedimentable by means of high-speed centrifugation have been isolated from most if not all of the normal and neoplastic plant and animal tissues studied hitherto (5, 32) and recently from a yeast (30) and a bacterium (31). Claude has found (5, 30) that sedimentable materials derived from many normal tissues are complex chemical entities containing large proportions of alcohol-soluble materials (phospholipids) as well as nucleoprotein of the ribose type. When the chemical find

ings are considered in relation to what is known about cellular antigens (33, 37), it does not seem surprising that the alcohol-soluble fractions should contain substances giving the Wassermann and Forssman reactions, and that the protein components should bear the stamp of the species from which they are derived, as Furth and Kabat have demonstrated (5)

Henle, Chambers, and Groupé (5) have recently cited reasons for supposing that the sedimentable cell constituents may possess distinctive organ-specific characters and have sought evidence for these by injecting the sedimentable substances of various tissues and species into rabbits. The findings of Tables X, XI, and XII of the present paper do not necessarily imply, by contrast, that the sedimentable constituents of various tissues lack organ-specific properties, they indicate, however, that the natural antibody does not distinguish any such. In this relation the fact deserves mention that, with a few noteworthy exceptions, it has proved difficult if not impossible to define organ-specific substances by ordinary serological methods (33, 36), special techniques,—such as those employed in the work with nephrotoxins (34) and in the demonstration of passive anaphylaxis (35),—have usually been required to achieve this end

In the experiments of the present paper, sera and tissues of the same species (sometimes of the same individual) were used, and immunization procedures were excluded, in order to avoid species- and presumably organ-specific effects. Hence it seems significant that the material with which the natural antibody reacts appears to be the same or closely similar whether derived from one or another of widely various tissues (Tables X, XI, and XII). Several kinds of "heterogenetic" substances are widespread in nature (33), this being true especially of the substance (or substances) that reacts with Wassermann reagin (11), yet the substance with which the natural tissue antibody reacts differs notably from the "heterogenetic" substances heretofore described. For it does not come away into alcohol as many of these do, it is unstable upon standing in saline solution and is destroyed by an amount of heating that has no deleterious effect upon the other substances mentioned (see Table IX), furthermore, though present in many normal tissues, it has not been detected in non-nucleated erythrocytes or in skin. Its properties, viewed as a whole, suggest that it may be a protein. Whether it will prove identical with one or another of the enzymes common to many tissue cells is a problem of immediate interest.

The Sedimentable Constituents of Normal Tissues and the Distinctive Substance of the Brown-Pearce Carcinoma

The sedimentable constituents of normal tissues had not been recognized when the observation was made that the Brown-Pearce tumor contains such a material and that this is distinctive in character and reacts specifically with an antibody which is present only in the blood of animals implanted with the tumor (1). But other sedimentable substances—the viruses—were known then

to be antigenic, and the antigen procured from the Brown Pearce tumor was found to have several properties strikingly similar to those of certain viruses,—notably its comparatively large particle size and weight as determined by ultrafiltration and ultracentrifugation and its reaction to changes in pH and to heat. When studied serologically its resemblance to the rabbit papilloma virus (Shope) seemed especially noteworthy, though the two proved wholly distinct from one another, and the Brown Pearce substance has proved non pathogenic upon inoculation into normal animals under a variety of conditions (1, 38) Whether the distinctive substance of the Brown Pearce tumor resembles more the sedimentable constituents of normal tissues than it does the viruses is a question that may be left to the future. In any case its specificity is manifest (see Tables XIII and XIV) More will be said about it in forthcoming papers, in relation both to the normal tissue constituent herein described and to another distinctive sedimentable substance which has recently been found in the V2 carcinoma, a transplantable tumor derived from a virus-induced rabbit papilloma (39)

SUMMARY

Continued serological investigations of the sedimentable constituents of normal and neoplastic tissues have shown that the blood serum of normal rabbits will fix complement in mixture with saline extracts of normal rabbit tissues. The phenomenon has proved referable, not to anticomplementary effects of serum or antigen nor to so called non-specific complement fixation, but to a naturally occurring serum principle, hitherto unrecognized which reacts specifically *in vitro* with a sedimentable constituent of normal tissue cells

The principle exists in the blood of nearly all adult rabbits but is absent from that of rabbits less than 1 month old. It can be salted out from serum with ammonium sulfate and is destroyed when heated at 65°C for 20 to 30 minutes. Its titer was found to run parallel in general with that of two natural antibodies also present in normal rabbit's blood (natural Wassermann reagin, natural anti sheep hemolysin) but absorption tests showed it to be distinct from these. Because of its properties, the serum principle has been termed the natural tissue antibody

The substance with which the natural tissue antibody reacts is regularly present in saline extracts of many normal tissues—those of rabbits and of other species as well. Kidney and liver tissues always yield it in abundance, while spleen, brain, and testicle provide somewhat less heart and voluntary muscle extracts contain relatively little, and non-nucleated erythrocytes and skin are practically devoid of it. The results of affinity and absorption tests indicate that it is nearly or quite the same from whatever tissue or species derived. It is readily sedimentable in the high speed centrifuge little or none

remaining in the supernatant liquid of potent suspensions spun at 25,000 R P M (45,400 g) for 1 hour. It either does not come away into alcohol or is inactivated thereby, is readily destroyed by heat (56–70°C for 30 minutes), and diminishes notably in antigenic potency upon standing overnight in saline suspension or when the tissues containing it are kept in glycerol. Its properties suggest that it may be a protein.

The implications of the findings are discussed in relation to the formation of the natural antibody and its place amongst serological phenomena, to so called "non-specific" fixation of complement and other serological complexities, and with particular reference to the character of the sedimentable constituents of normal and neoplastic tissue cells.

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THE METABOLISM OF THE CENTRAL NERVOUS SYSTEM IN EXPERIMENTAL POLIOMYELITIS*

By E. RACKER, M.D., AND HERMAN KABAT, M.D. †

*(From the Anderson Institute for Biological Research and the Department of Physiology,
University of Minnesota, Minneapolis)*

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Little is known of the mechanism of destruction of neurons by the virus of poliomyelitis. The older theory (1) that neurons of the spinal cord are injured as a result of impaired circulation is apparently not widely supported at present. Significant evidence has accumulated that the virus spreads through neurons (2) and that the mesodermal-glial changes are secondary to the involvement of nerve cells (3). It seems probable that the virus damages the neuron directly by its presence in the cell, disrupting some vital cellular function.

However, the mere presence of virus in a neuron does not signify that the cell will be destroyed. Poliomyelitis virus has been shown to spread through many neurons in the brain without producing cellular necrosis (4). Reversible neuronal injury may account to some extent for the recovery from paralysis frequently observed in clinical cases of the disease. The local pathological lesion produced by injection of poliomyelitis virus into the visual cortex in the monkey is insignificant in comparison to the reaction around a similar inoculum in the motor cortex (3). Recently, Bodian and Howe (5) have made the striking observation that highly susceptible neurons of the anterior horn of the spinal cord may, by interruption of their axons, be protected from poliomyelitis virus.

It is reasonable to suppose, as a working hypothesis, that the virus of poliomyelitis may interfere with certain specific metabolic activities of the nerve cell, and that the relative importance of these activities to a particular neuron may determine resistance or susceptibility to the virus. In beginning to explore this hypothesis, an investigation has been made of some aspects of the metabolism of the central nervous system of mice infected with poliomyelitis virus. In addition, normal tissues from regions of the central nervous system which have been shown to differ markedly in susceptibility to the virus have been examined to determine their metabolic activities.

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Methods

Swiss albino mice 4 to 6 weeks of age were inoculated intracerebrally with 0.03 cc. of a 10 per cent suspension of brain tissue from mice infected with the Lansing strain of poliomyelitis virus. When definite paralysis became evident, the animal was sacrificed, the brain removed, weighed, dispersed in a mortar or finely minced, and suspended in Locke's solution to make a 10 per cent suspension. All was done as rapidly as possible. In some instances, marked symptoms of encephalitis were present but in no case was the mouse moribund at the time it was sacrificed. A suspension of normal mouse brain was prepared at the same time and in an identical manner, serving as a control.

TABLE I
Metabolism of Mouse Brain Suspensions

Batch	Oxygen utilization					Anaerobic glycolysis				
	Normal		Poliomyelitis			Normal		Poliomyelitis		
	No. of mice	O ₂ uptake*	No. of mice	O ₂ uptake*	Deviation from the normal	No. of mice	CO ₂ † output	No. of mice	CO ₂ † output	Deviation from the normal
					per cent					per cent
1	5	92.8	3	97.1	+4.6	5	51.6	5	27.1	-47.5
2	4	120.4	4	119.9	-0.4	6	75.5	6	63.0	-16.5
3 and 4	5	86.5	4	86.2	-0.3	19	87.9	12	74.1	-15.7
5	8	98.9	6	104.0	+5.1	14	100.5	11	93.1	-7.3
6	5	71.2	6	78.8	+10.6	5	71.2	5	60.8	-14.5
7	8	87.3	8	80.0	-8.4	7	75.4	7	70.4	-6.6
8	3	128.6	3	107.6	-16.3	5	94.8	5	74.4	-21.5
Total	38	95.0	34	93.4	-1.6	61	84.4	51	70.5	-16.5

* Average of oxygen consumption in c.mm. per 100 mg. wet weight in first hour

† Average of CO₂ output in c.mm. per 100 mg. wet weight in first hour

Oxygen utilization and anaerobic glycolysis of normal and poliomyelitic brain suspensions were measured by the usual manometric methods at 37°C and pH 7.4, using the Barcroft-Warburg apparatus. Approximately 100 mg. wet weight of brain tissue was introduced into each vessel. In some instances, slices of mouse cerebrum were used and dry weight determined at the end of the experiment. For oxygen consumption, a phosphate glucose-Locke medium was used, for anaerobic glycolysis, a bicarbonate-glucose-saline solution was used and the system filled with 95 per cent N₂ and 5 per cent CO₂.

RESULTS

The results of investigation of the oxygen utilization and anaerobic glycolysis of suspensions of surviving brain tissue from normal and poliomyelitic mice are tabulated in Table I. There was no significant difference in oxygen consumption of normal and infected brain with glucose as the substrate. This

was confirmed in experiments on slices of cerebrum the average Q_{O_2} for normal brain was 10.4 and Q_{O_2} for poliomyelitic brain was 9.9. This is in conformity with the observations of Brodie and Wortis (6), who found no difference in oxygen consumption of normal and poliomyelitic spinal cord from monkeys. No difference in oxygen consumption by normal and poliomyelitic brain tissue was noted in experiments using other substrates, such as lactate, pyruvate, succinate, and glycogen. This was true even when these substrates were tipped into the vessel at the end of the third hour, allowing endogenous substrates to be depleted.

In contrast to the results on oxygen consumption, a consistent and significant decrease in anaerobic glycolysis was observed in tests of poliomyelitic brain suspension (Table I). The average values from a large number of experiments showed a percentage decrease of 16.5 per cent from the normal. The decrease was noted in practically every case although it varied in degree from 5 per cent to over 50 per cent in individual experiments. The percentage decrease in anaerobic glycolysis in the infected brain varied considerably for different batches of mice of the same age and strain which may be related to the marked differences in susceptibility to poliomyelitis observed with different strains of Swiss mice (7). It was noted that the decrease of glycolysis was usually much greater when symptoms of encephalitis had been evident before the mouse was sacrificed, while simultaneously measured oxygen utilization remained unchanged. The wide variations in extent and severity of brain lesions in rodents infected with the Lansing strain of poliomyelitis virus (8) may help to explain the individual variations in the decrease of anaerobic glycolysis in infected brain tissue.

Suspensions of cerebrum, brain stem minus cerebellum, and spinal cord from four poliomyelitic mice were compared with the same regions of the central nervous system from normal mice. The inhibition of anaerobic glycolysis in cerebrum and brain stem was over 30 per cent, while in spinal cord, the inhibition was slightly less.

In an attempt to find the reason why the anaerobic breakdown of glucose in brain is less in poliomyelitis, experiments were performed using sodium fluoride as an inhibitor (Table II). The percentage inhibition resulting from fluoride was consistently higher in the normal than in the infected brain, particularly with lower concentrations of fluoride. The residual glycolysis in poliomyelitic brain treated with fluoride was equal to the glycolysis in normal brain with fluoride in every experiment. One of the major effects of fluoride is to inhibit glycolysis at the stage of breakdown of phosphoglycerate to phosphopyruvate. In contrast to the results with fluoride preliminary experiments indicate that monoiodoacetate exerts an equal inhibitory effect on anaerobic glycolysis of normal and poliomyelitic brain suspensions.

Experiments were also performed to compare the dehydrogenase activity

of normal and poliomyelitic brain suspensions. The technique used was the Thunberg method of determination of rate of decolorization of methylene blue in an evacuated tube. Without added substrate, the methylene blue was uniformly decolorized more rapidly by the infected than by the normal tissue. The average decrease in decolorization time was 20 per cent, but was much higher in individual experiments. On the other hand, with addition of various substrates, for example glucose, lactate, and succinate, there was no significant difference observed between normal and poliomyelitic brain. It appears possible that the more rapid decolorization by the infected tissue may be related to the presence of a greater quantity of available substrate, due to changes in the chemical composition of the brain resulting from poliomyelitis. No quantitative correlation could be observed between the increase in dehydrogenase activity and the decrease in anaerobic glycolysis in infected brain.

TABLE II
The Effect of Sodium Fluoride on Anaerobic Glycolysis

Concentration NaF	Normal				Poliomyelitis			
	No. of mice	Without NaF	With NaF	Inhibition	No. of mice	Without NaF	With NaF	Inhibition
				per cent				per cent
M/30	6	66.5*	8.9	86.6	7	56.9*	12.6	77.8
M/60	1	86.4	9.1	89.4	1	78.6	11.7	85.1
M/150	3	100.3	27.9	72.1	3	78.9	32.3	59.0
M/300	1	111.3	40.6	63.5	1	87.7	41.9	52.2

* CO₂ output in c.mm. per 100 mg. wet weight of brain per hour

Preliminary observations have been made on the phosphatase activities of normal and poliomyelitic mouse brain suspensions, measured at pH 5.5 and pH 9.5, using veronal buffer. The substrates tested were β -glycerophosphate, nucleic acid isolated from mouse brain tissue, and adenosine triphosphate. The suspension was incubated for 2 hours at 37°C. in a shaking apparatus. Inorganic phosphate was determined colorimetrically. Although the number of experiments was small, the phosphatase activity of the infected brain appeared to be higher with mouse brain nucleic acid and adenosine triphosphate as substrates, while no difference from normal was noted with β -glycerophosphate as the substrate.

In an attempt to determine whether specific susceptibility of different brain regions to the virus of poliomyelitis might be related to differences in metabolism of these regions, a comparison has been made of oxygen consumption and anaerobic glycolysis of the motor cortex, visual cortex, and anterior horn of the spinal cord from normal animals. Bodian and Howe (3) have demonstrated in the monkey that visual cortex is much less susceptible than motor

cortex to inoculation of poliomyelitis virus, and it is well known that anterior horn cells are highly susceptible to this virus.

The technique used in these experiments was as follows—

Dogs and cats were anesthetized with pentobarbital sodium the calvarium carefully removed. In some cases motor cortex and in other instances visual cortex was excised and quickly sliced and washed in the medium containing glucose. The remaining cortex (visual or motor in different experiments) which had remained *in situ* with adequate circulation, was then excised and sliced in the same manner. In this way the slices were obtained as fresh as possible. Slices of anterior horn of the lumbo-

TABLE III
Metabolism of Different Regions of the Central Nervous System
(Normal Dog and Cat)

Motor cortex			Visual cortex			Normal anterior horn			Chromatolytic anterior horn		
Q_{O_2}	Q_{CO_2}	$\frac{Q_{CO_2}}{Q_{O_2}}$	Q_{O_2}	Q_{CO_2}	$\frac{Q_{CO_2}}{Q_{O_2}}$	Q_{O_2}	Q_{CO_2}	$\frac{Q_{CO_2}}{Q_{O_2}}$	Q_{O_2}	Q_{CO_2}	$\frac{Q_{CO_2}}{Q_{O_2}}$
-6.4	+7.9	1.23	-6.9	+4.0	0.58	-1.7	+1.4	0.82	-1.4	+1.9	1.36
-5.0	+4.0	0.8	-6.6	+4.0	0.60	-3.0			-2.5		
	+4.0			+4.1		-2.0			-2.0	+1.7	0.85
-7.3			-6.3				+2.0				
	+2.7			+1.3		-4.3	+4.7	1.09			
-7.9			-9.0								
-10.7			-9.5								
-10.2			-8.1								
-7.8	+8.5	1.1	-8.0	+4.2	0.52	-3.9	+1.9	0.49			
-5.8	+5.0	0.86	-6.4	+3.3	0.51						
-6.0	+7.4	1.2	-5.9	+3.3	0.56						
Average											
-7.4	+6.2	1.04	-7.4	+3.4	0.55	-3.0	+2.5	0.80	-2.0	+1.8	1.1

sacral spinal cord were obtained in a similar fashion. The Q_{O_2} and Q_{CO_2} were determined in the usual manner using the Barcroft Warburg apparatus. It is now well established that barbiturates can be quickly removed from brain slices by washing (9) and do not exert a lasting effect on metabolism. While dog and cat are admittedly insusceptible to the virus of poliomyelitis, it is not known whether this resistance is based on immunological or chemical rather than metabolic factors.

The results of the experiments on slices of motor and visual cortex and anterior horn of the spinal cord are tabulated in Table III. There is apparently no difference in oxygen consumption between motor and visual cortex, while the anaerobic glycolysis is significantly lower for visual cortex. The oxygen utilization of anterior horn is only a fraction of that of cortical tissue. A few

experiments on chromatolytic anterior horn tissue obtained from dogs subjected to section of the sciatic and femoral nerves 3 weeks previously are also included, since chromatolysis greatly influences susceptibility of anterior horn cells to poliomyelitis. One must be cautious in interpretation of the results on anterior horn slices because of the low metabolic activity of this tissue. Of particular interest is the ratio Q_{CO_2}/Q_{O_2} , which is consistently higher for motor cortex than for visual cortex. Whether these results have any bearing on susceptibility of specific regions to poliomyelitis virus is still problematical.

DISCUSSION

The only previous study of metabolic activity of surviving nervous tissue infected with poliomyelitis virus was reported by Brodie and Wortis (6), who found that minced brain and spinal cord from infected monkeys showed no significant change from normal in oxygen consumption or respiratory quotient. It may be noteworthy that their data show a decrease in lactic acid content of the poliomyelitic brain.

All investigators agree that purified viruses do not utilize oxygen or carry out dehydrogenase activity (10-14). Phosphatase activity has been observed in bacteriophage (12), and tobacco mosaic virus (13), and phosphatase and catalase activities have been reported for elementary bodies of vaccinia (14). Of interest are the recent studies demonstrating the presence of biotin (15), copper (16), and flavin (17) in purified vaccinia virus.

SUMMARY

1 During paralysis, the brain of the mouse infected with poliomyelitis virus shows on test after mincing a decrease in anaerobic glycolysis with no significant change in oxygen utilization. The decrease in anaerobic glycolysis varies from 5 per cent to 50 per cent.

2 Sodium fluoride produces a greater inhibition of anaerobic glycolysis in normal than in poliomyelitic brain.

3 Dehydrogenase activity is higher for poliomyelitis-infected brain without added substrate. This difference from normal disappears when substrates are added.

4 The ratio of $\frac{\text{Anaerobic glycolysis}}{\text{Oxygen utilization}}$ for the sliced motor cortex is higher than for sliced visual cortex of the dog and cat.

5 The oxygen consumption of the anterior horn of the sliced spinal cord of dog and cat is much less than that of the cerebral cortex.

6 The findings are in keeping with the view that, at a certain stage of the infection, the nerve cells may be reversibly injured but not yet destroyed by the virus.

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